DEVELOPMENT AND EVALUATION OF HERBAL OINTMENT FROM *Justicia procumbens* Linn., FOR INFLAMMATION ON EYE

A Dissertation submitted to

THE TAMILNADU Dr. M.G.R MEDICAL UNIVERSITY

CHENNAI-600 032

In partial fulfilment of the requirements for the award of degree of

MASTER OF PHARMACY

IN

PHARMACOGNOSY

Submitted by

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MAY 2017

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This is to certify that the dissertation entitled " **DEVELOPMENT AND EVALUATION OF HERBAL OINTMENT FROM *Justicia procumbens* Linn., FOR INFLAMMATION ON EYE** " submitted by **Reg. No: 261520651** in partial fulfilment of the requirements for the award of the degree of **MASTER OF PHARMACY IN PHARMACOGNOSY** by The Tamil Nadu Dr. M.G.R Medical University, Chennai, is a bonafide record of work done by him in the Department of Pharmacognosy, College of Pharmacy, Madras Medical College, Chennai-600003, during the academic year 2016-2017 under the guidance of **DR.R.VADIVU, M.PHARM., Ph.D., Asst.Professor,** Department of Pharmacognosy, College of Pharmacy, Madras Medical College, Chennai-600003.

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1.1 INTRODUCTION [1]

Allergic conjunctivitis is an undesirable inflammatory reaction of the conjunctiva resulting from acquired immune response against an allergen (a foreign antigen to which the individual has been previously sensitized). The conjunctiva is a thin transparent outermost membrane lining the eyeball surface. It is richly supplied by blood and easily gets inflamed when exposed to adverse environmental conditions.

Allergic conjunctivitis is estimated to affect about 15% of the world‘s population with variations observed among different geographical areas and age groups. Studies show that ocular symptoms of allergic conjunctivitis are presented in about 50% of individuals suffering from other allergic disorders.

A survey among adults in the United States revealed that about 40% of the respondents experienced recurrent symptoms of ocular itch and tearing which are characteristic of ocular allergy.

A Tanzanian study was carried among 400 households in which was found that more than half of the households had one or more family members suffering from allergies and ocular allergy alone accounting for 10.8% of the total prevalence.

1.2 ALLERGIC CONJUNCTIVITIS [1]

Allergic conjunctivitis (AC) is a collective term used to describe diverse ocular allergic disoders including seasonal allergic conjunctivitis (SAC), perennial allergic conjunctivitis (PAC), vernal kertoconjunctivitis (VKC), atopic keroconjunctivitis (AKC), gaint papillary conjunctivitis (GPC) and contact dermatoconjunctivitis (CDC). All these forms of allergic conjunctivitis can be broadly categorized into 2; namely acute allergic conjunctivitis and chronic allergic conjunctivitis depending on the underlying immunological mechanism.

1.2.1 Acute Allergic Conjunctivitis

SAC and PAC are acute forms of AC caused by immediate hypersensitive response in eyes of sensitized individuals after subsequent exposure to an already sensitized agent (allergen). Ocular itching is a charateristic symptom of AC and is often accompanied with tearing. Common signs observed are conjunctival hyperemia and chemosis. Mucoid discharge, conjunctival papillae, red edematous eyelids and pseudoptosis are found in pronounced cases.

1.2.2 Chronic Allergic Conjunctivitis

Atopic keroconjunctivitis and Vernal kertoconjunctivitis are chronic forms AC caused by both immediate and delayed hypersensitivity. Patients present with similar history of allergies (personal or familial) as in the acute forms. The onset of VKC is in childhood and has a predilection for males and the African race, and usually resolves spontaneously without complications in the late teens if well managed. Sufferers of VKC experience remission of ocular symptoms during cold seasons while AKC has no particular seasonal predilection. A significant sign aiding in the differential diagnosis of

AKC from VKC is the component of periocular skin and lid changes which manifest as scaling and flaking of the lids.

GPC and CDC are also chronic and caused by persistent mechanical irritation and adverse reaction to topical medications or cosmetic products. The rationale in categorizing these conjunctival reactions under ocular allergic disease is due to evidence of an increased number of mast cells in the conjunctiva and increased levels of IgE in the tear film of patients.

1.2.3 Factors Associated to Allergic conjunctivitis [2]

The increasing trend observed in the prevalence of allergic conjunctivitis is alarming and has led to investigations to provide insight and understanding into the development of this disorder. Researchers have identified several risk factors some genetic and others environmental inclined.

1. Family History

2. Gender

Males are more susceptible to VKC than females.

3 Socioeconomic status

4 Breastfeeding

Infant as it serves as an irreplaceable passive and active immunity

5 Childhood use of antibiotics

6 Obesity

7 Nutrition

8 Stimulant use

The use of stimulants like alcohol and drugs are investigated for their role in many diseases.

9 Lack of microbial exposure (hygiene)

10 Smoking

some researchers have shown that smoking is associated with increased total IgE levels

1.3 THE IMMUNE SYSTEM [1-3]

An allergic response is a consequential adverse inflammatory effect that accompanies the immune system‘s defense that is mounted to the eradicate substances which are considered foreign from the body. The defensive systems of the body is grouped into 2 namely; the innate immune system and the acquired immune system.

1.3.1 Innate Immunity

The innate immune system is fully present and functional at birth and is the host‘s first line of defense against invading pathogens. Innate immunity is mounted by anatomical structures including the skin, nasal and conjunctival mucosal surfaces, cilia and other phagocytic cells (blood monocytes, neutrophils and tissue macrophages). Other physiological responses such as fever and low pH also play a key role in inhibition of microbial growth.

1.3.2 Acquired Immunity

The acquired immune system comprises two main branches; the humoral immunity and the cell mediated immunity. The acquired immune system is however not fully active at birth and requires prior exposure to an antigen (foreign substance) either by infection or immunization. Emphasis is laid on the humoral immune system which is crucial in the development of the immediate hypersensitivity.

Upon the first encounter of an individual to an antigen results in a relatively weak, short-lived response called the primary immune response which is characterized by the

production of plasma cells (antibody-secreting cells) and memory B cells. It takes averagely about 1 week from the time of contact to the offending agent to the realization of antibodies circulation. This period from exposure till when antibodies are produced is referred to as the Latent phase.

1.3.3 Cellular Components of the Immune System

The cells of the immune system are basically those cellular components of blood referred to as the white blood cells. These include the granulocytes (neutrophils, eosinophils, basophils), macrophages, dendritic cells and mast cells all differentiating from the myeloid progenitor cell. The lymphoid progenitor cells also differentiate into the three lymphocytic cells; namely the T cells, B cells and NK cells. These two forms of progenitor cells originate from the hematopoitic stem cells in the bone marrow. All these cells play a crucial role in the defense of the body against antigens. However, the main cells that participate in allergic reactions are the dendritic cells and macrophages (referred to as antigen presenting cells), lymphocytes (which include the T cells and B cells), mast cells, basophils and eosinophils.

1.3.3.1. Antigen Presenting Cells

Antigen presenting cells (APCs) are those cells of the immune system that express MHC II molecules on their surface and are capable of recognizing antigens, and then process the antigens through phagocytosis after which they are presented for the activation of a naïve T cell. The phagocytozed antigens are presented as peptide fragments bound to a class II MHC molecule, on the membrane. This antigen-class II MHC complex binds to T cell receptor (TCR) and this together with other co-stimulatory molecules expressed by the APC activates the T cell.

1.3.3.2 Lymphocytes

Lymphocytes are white blood cells which are very important components of the adaptive immune response. There are three major types of lymphocytes-T cells, B cells and NK cells, and all these cells are derived from the bone marrow but mature at different locations. The T cells mature in the thymus, the B cells in the bone marrow and the NK cells in the primary (bone marrow and thymus) and secondary lymphoid tissues (spleen, lymph nodes, tonsils, and mucosa-associated lymphoid tissue).

1.3.4 Chemical Mediators of the Immune System [3]

1. Histamine

2.Prostaglandins

3. Leukotrienes

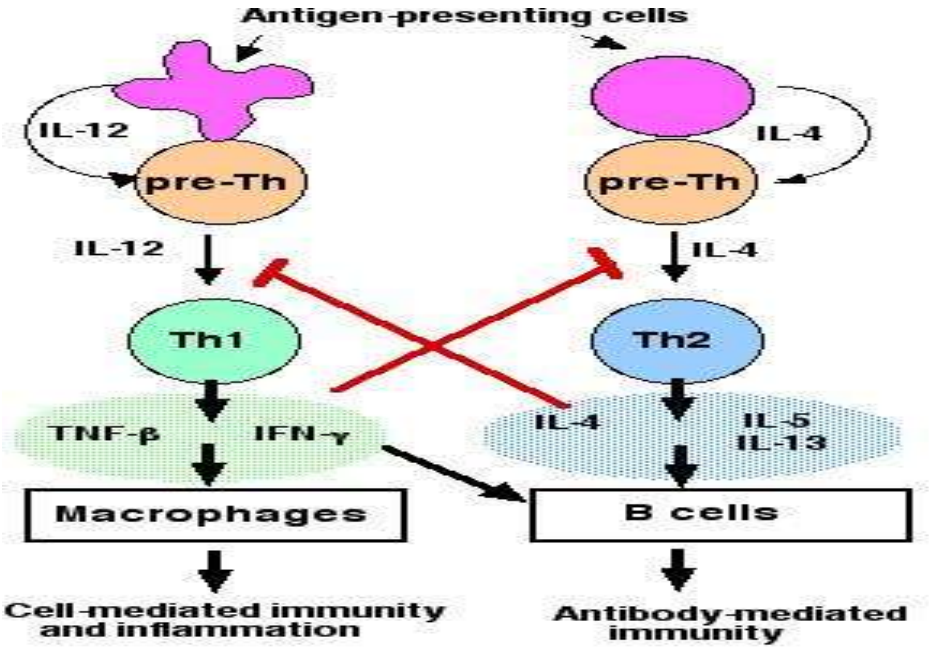

Figure 1: Th1/Th2 balance. Depending on the cytokine milieu, pre-Th cells may develop to Th1 or Th2-cells that inhibit each other and drive cell-mediated immunity or humoral immunity responses.

1.4 PATHOGENESIS OF OCULAR ALLERGY [3,4]

Allergic conjunctivitis is an immediate hypersensitive response of the conjunctiva and is Th2 cell driven culminating in the production of IgE by plasma cells which mediate the inflammatory process. The sequence of events leading to an allergic reaction is as described. When an allergen intrudes the body, it is bound by APC and presented as antigen-MHC-II complex molecule to a naïve T Cells.

An interaction is initiated between a protein called the CD40 Ligand found on the surface of the Th2 cell, and the CD40 protein on the B-cell surface resulting in activation of the B cell. In addition, Th2 cell also secretes cytokines (IL-4, IL-5 and IL-13) serving as co-stimulatory molecules in the B cell activation influencing the class-switching of B cells to IgE synthesis and the recruitment of mast cells. Large amounts of IgE antibodies could be produced over several years and in the complete absence of allergen. Once formed and released into the circulation, IgE binds, through its Fc portion, to high affinity receptors (FCeRI) on mast cells, basophilic and eosinophilic granulocytes, leaving its allergen specific receptor site available for future interaction with an allergen. The process from the exposure to the allergen until the binding of IgE to the high affinity receptors is referred to as sensitization . Although, serum concentration of IgE is low (i.e. 0.05mg/mL), its binding to high affinity receptors prevents it from proteolytic cleavage and clearance which occurs in unbound IgE in circulation. Mast cell-bound IgE then remains in tissues for a long time and have the potential to react immediately to a specific allergen.

1.5 DIAGNOSTIC PROCEDURES [3-6]

1.5.1 Clinical Diagnosis

A typical diagnosis of ocular allergy is usually made in the clinic during physical examination of patients. Ocular symptoms including tearing, redness, ropy discharge, burning, pain and swollen lid could be reported. The clinician then performs assessment for signs such as conjunctival redness, chemosis, lid edema, papillae et cetera. However, occasionally it may be necessary to perform some additional laboratory testing either to support the clinical diagnosis or to identify the causative agent to ensure an effective management strategy.

1.5.2 Serum IgE Tests

The test measures the concentration of IgE antibodies in the blood, which could be the total IgE tests or antigen-specific IgE (s-IgE). Although an elevated total IgE level in serum is frequently found in atopic individuals, it could also be found in non-atopic individuals. However, s-IgE tests can be especially useful when SPTs cannot be done (examples are presence of skin disorders and usage of antihistamine medications). Two common tests based on this principle are the radioallergosorbent test and, the much sensitive test, enzyme-labeled immunosorbent assays.

1.5.4 Tear IgE measurements (Lacrytest)

Lacrytest is a qualitative and a rapid immunoassay procedure for determining whether or not the total tear IgE level is above the normal value (<2KU/L, 3ng/mL) observed in healthy subjects. This requires placing a strip in the lower conjunctival fornix to be wet with tears. Total IgE reacts with a gold-labelled antibody and is immobilised with the uptake of anti-IgE antibody. Signal intensity is dependent on the

total IgE. For normal values, below 2.5 KU/L, no line is obtained.

1.5.5 Atopy patch tests

The atopy patch test is of significant importance in the diagnosis of AKC and CDC. The procedure involves epicutaneous application of adhesive patches impregnated with suspected allergens. This is followed by evaluation for eczematous skin lesions at least twice, usually at 48 hours after application of the patch and again two or three days later.

1.5.6 Conjunctival Provocation Test (CPT)

This test incorporates both diagnostic and therapeutic protocols and requires topical instillation of serial dilutions of suspected allergen determined by clinical examination and SPT into one eye and the other eye (control) instilled with balanced salt solution. Slit- lamp examination of eyes is done at different times. Criteria for a positive test are congestion of the conjunctival mucosa, itching, and eye watering. After the ocular allergy has been induced, topical antihistamine is applied to control allergic reaction. CPT therefore serves as a clinical protocol for inducing ocular allergy or determining ocular response to allergens, and evaluation of efficacy of anti-allergic medications.

1.6 CURRENT TREATMENT AND CLINICAL TRIALS [6-8]

Management of allergic conditions can be categorized broadly into non-pharmacological and pharmacological treatment.

1.6.1 Non-pharmacological treatment

Evidence indicates that allergic conjunctivis could be effectively managed by

relying on prophylactic measures. Mechanical rubbing of the eyes lead to mast cell degranulation and further release of inflammatory mediators and individuals are normally discouraged from this act.

1.6.2 Pharmacological treatment

For a sensitized individual, if avoidance of the allergen could not be achieved and the conjunctival mucosa (physical barrier) is traversed, then there is the need to prevent the acquired specific immune response which is antibody-mediated. Intervention at this stage usually requires the usage of pharmacological (anti-allergic) agents

The general mechanisms of action of anti-allergic medications can be broadly categorized as: 1) agents inhibiting synthesis or release of the chemical mediators (such as histamine, prostaglandins and leukotrienes), and 2) agents inhibiting chemical mediators from their target sites.

1. Mast Cell Stabilizers
2. Antihistamines
3. Steroids
4. Non-steroidal anti-inflammatory drugs (NSAID)
5.Leukotriene Antagonists

Herbal plants used for ocular inflammation

Since herbal plants are obtained naturally they exhibit a lot of goodness rather than side effects as like in cases of allopathic medicinal systems which compounded by humans.
So we more prompt to choose herbals for its less effects.

Aloe vera (Linn.) Burm.f. (Liliaceae) Ghrit kumara-The leaf pulp is applied externally to cure conjunctivitis. One to two drops of squeezed material is applied in both the eyes to get soothing relief from pain and inflammation.

Amomum subulatum Roxb. (Zingiberaceae)

Cardamom-Three drops of oil extracted from seed is applied three to inflamed eyelids daily to cure conjunctivitis.

Azadriachta indica A. Juss. (Meliaceae) Neem -The poultice of leaf are applied on eye lid twice to cure conjunctivitis.

Cassia auriculata Linn. (Caesalpinioideae)

Aavartoki- Presoaked seed are made in to paste. The paste is applied thrice over inflammed eyes to cure conjunctivitis.

Emblica officinalis Gaertn. (Euphorbiaceae)

Amla-The fruit sauss mixed with honey and taken only twice a day to improve weak eye sight. It is also an important constituent of triphla which is known for various eye ailments.

Justicia procumbens Linn., (Acanthaceae)

Leaves are juiced and applied on to the eye for ophthalmia cases.

Ocimum tenuiflorum Linn., (Lamiaceae)

Dhulungshi-The leaf juice is used for eye disease.

Crocus sativus Linn.,(Iridaceae)

Two to three drops are applied thrice to relief irritation and cure conjunctivitis.

Tamarindus indica Linn. (Caesalpiniaceae)

Imli-Two - three drops of leaf juice is applied on eyes thrice a day to cure inflammations, redness and water discharge due to conjunctivitis

Tectona grandis Linn. (Verbenaceae) Teak-Ashes of wood are applied over swollen eyelids to cure conjunctivitis

Camellia sinensis (Linn.) Kuntz (Theaceae)

Tea, chay-Two-Three drops of leaf juice is applied in eyes thrice daily to cure conjunctivitis.

Cissampelos pariera Linn. (Menispermaceae)

Puranpadi, Akandi-Leaf juice is used in case of inflammed eyes so as to cure conjunctivitis.

In order to identify the plant which is traditionally used in the treatment of conjunctivitis, a thorough literature survey was carried out on plants used traditionally for treating conjunctivitis. Although many plants are used to treat conjunctivitis in indian medicinal traditional systems of medicine, most of these plants are not scientifically evaluated. If a systemic and intensive ethno pharmacological study is carried on one or more plants used in traditional system, are sure to provide effective drug for treating conjunctivitis.

2. REVIEW OF LITERATURE

A thorough literature survey was carried out in-order to identify the plant which is traditionally used in the treatment of inflammation of eye. Among so many herbs available to treat inflammation of eye *Justicia procumbens* was selected for the present study because it is traditionally used to treat inflammation of eye but not yet proven scientifically.

Literature review of *Justicia procumbens* Linn., was carried out to find out the research work carried out on this plant. The review of literature showed only little work were done on different part of this plant. This review is made to confirm the usefulness of this plant for treating inflammation of eye was not yet proven scientifically. Hence further studies were easy to carry out.

PHARMACOGNOSTICAL REVIEW

- Raj Kumar N *et al.,* (2009) studied ethnomedicinal application of plants in the eastern region of Shimga District, Karnataka, India.[11]
- N.Savithramma *et al.,* (2007) studied ethnobotanical survey of plants used to treat asthma in Andhra Pradesh, India[12]
- Miao-Ling Lin (2004) studied formation of Calcium Carbonate Deposition in the Cotyledons during the Germination of *Justicia procumbens* L.(Acanthaceae) Seeds.[13]
- Joshi K *et al.,* (2000) Indigenous knowledge and uses of medicinal plants by local communities of Kali Ghandaki watershed area, Nepal.[14]

PHYTOCHEMICAL REVIEW

- Luo *et al.,*(2013) studied Simultaneous determination of seven lignans in *Justicia procumbens* by high performance liquid chromatography-photodiode array detection using relative response factors.[15]
- Wang, L *et al.,* (2011) carried out Chromatographic fingerprint analysis and simultaneous determination of eight lignans in *Justicia procumbens* and its compound preparation by HPLC-DAD.[16]
- Su C.L *et al.,* (2006) proved Caspase-8 acts as a key upstream executor of mitochondria during justicidin A-induced apoptosis in human hepatoma cells.[17]
- Weng *et al.,* (2004) Isolated two new arylnaphthalide lignans and antiplatelet constituents from *Justicia procumbens*.[18]
- Lin Y C *et al.,* (2002) Isolated potent cytotoxic lignans from *Justicia procumbens* and their effects on nitric oxide and tumour necrosis factor production in mouse macrophages.[19]

- Chen CC *et al.,* (1998) Isolated six new diarylbutane lignans from Justicia procumbens[20]

- Fukamiya N *et al.,* (1986) Isolated antitumour agents – justicidin A and diphyllin two cytotoxic principles from *Justicia procumbens.* [21]

PHARMACOLOGICAL REVIEW

- K. Veeresh *et al.,* (2014) Studied antidiarrhoeal activity of methanolic extract of *Justicia procumbens* by castor oil and enterpooling induced methods in rats.[22]
- Chen CC *et al.,* (1996) Studied antiplatelet of arylnaphthalide Lignans from *Justicia procumbens* [23]
- Asano *et al.,* (1996) Studied antiviral activity of lignans and their glycosides from *Justicia procumbens* [24]

3. Plant Profile

The genus of *Justicia* comprises about 600 species that are found in pan tropical and tropical regions. It is procumbent, diffuse, slender, branching annual/perennial, 4- 16 inch in height, distributed in Bihar, West coast from Konkan to Kerala.

PLANT DESCRIPTION [9-12]

Water Willow is a slender, often tufted, prostrate or ascending, branched annual / perennial herb. The stems are 10-30 cm long, the leaves are elliptic to oblong-ovate or ovate, 7-20mm long, 5-20mm wide, obtuse at both ends and entire or slightly crenate margin.

The flowers are pink 6-7mm long and borne in terminal, rather dense cylindric spikes 1-5cm long and about 5mm in diameter. The bracts and calyx teeth are green, linear-lanceolate and hairy. The fruit (capsule) is slightly hairy and about 4mm long.

PLANT TAXONOMY[12]

Kingdom : Plantae – Plants

Class : Angiospermae

Subclasses : Eudicots

Order : Laminales

Family : Acanthaceae

Genus : *Justicia*

Species : *procumbens*

Synonyms

Rostellularia procumbens (L) Nees

Common names

Water Willow, Shrimp plant

Vernacular names

Tamil - Aarm, Kodaga salai, Kukurum, Kantheru

Malayalam - Cheriya oridalthamara

Hindi - Karambal

Konkani - Ghati pitpapad

English - Water willow

FIG 2 WHOLE PLANT OF *Justicia procumbens*


PLATE No. 725.
JUSTICIA PROCUMBENS, LINN.

TABLE 1 PLANT DESCRIPTION[13]

S.NO	PARTS	IMAGES	DESCRIPTION	CHEMICAL CONSTITUENTS	USES
1.	**Leaves**		Oblong-ovate, 7 - 20 mm long, 5 to 20 mm wide, obtuse at both ends, entire margin	Lignan glycosides, naphtha furanones,	Juice of the leaves is squeezed into the eye in cases of ophthalmia, treatment of wounds.
2.	**Flowers**		Pale purle in dense,6-7 mm long, borne in terminal, rather dense cylindric spikes 1-5cm long 5mm in diameter. Corolla 6-8mm.	Procumbenoside, Justicidinosides, Taiwanin, Neojusticin	Laxative, diaphoretic, diuretic, febrifuge
3.	**Fruits and seeds**		Slightly hairy, about 4 mm long. Capsules oblong, shortly pointed, pubescent at the tip; Seeds finely tuberculate , orbicular, brown, straitely rugose	Diphyllin, Justicidin	Asthma, cough, backache, plethora, flatulence

4. RATIONALE FOR SELECTION

Plants are used as food and as well as medicine in India. Many plants have been traditionally used in Siddha and Ayurveda medicinal systems to treat various disease and disorders. Although these plants are stated to have medicinal properties but they lack detailed scientific studies. In a desire to bring value and recognition to our traditional medicinal system *Justicia procumbens* Linn,. (Acanthaceae) was selected for the present study for treating conjunctivitis by developing an herbal eye ointment.

Only a handful of studies are done in the plant *Justicia procumbens* Linn,. Lack of Pharmacognostical, Phytochemical and Pharmacological studies makes this plant an ideal target for the scientific studies of traditionally used medicinal plants. This plant is less studied and has a great potential in medicinal properties.

- No study for Conjunctivitis is done in any part of this plant.
- This plant is claimed to be used in treating eye in case of ophthalmia in traditional medicinal system
- Hence, this plant is taken for the present study.

5.AIM AND OBJECTIVES

AIM

Development and Evaluation of Herbal ointment for inflammation of eye from the leaves of *Justicia procumbens* Linn.,

OBJECTIVES

Pharmacognostical studies

Establishment of Pharmacognostical standards for the leaves of *Justicia procumbens* Linn.,

Phytochemical studies

To identity the phytochemical constituents present in the plant.

Selection of active extract

To select the active extract by *in-vitro* anti-oxidant studies.

Development and Evaluation of herbal ointment

Pharmacological studies:

To evaluate the formulated herbal eye ointment by *in-vivo* method.

6.PLAN OF WORK

1. COLLECTION OF PLANT MATERIAL
2. AUTHENTICATION
3. PHARMACOGNOSTICAL STUDIES
    - ❖ Macroscopy
    - ❖ Microscopy
        - Transverse section
        - Powder microscopy
        - Histochemical studies
    - ❖ Physicochemical constants
4. PHYTOCHEMICAL STUDIES
    - ❖ Preparation of extracts
    - ❖ Preliminary phytochemical analysis
    - ❖ Quantitative estimation of phytoconstituents
    - ❖ Fluorescence analysis
    - ❖ Thin layer chromatography
    - ❖ High performance thin layer chromatography
5. SELECTION OF ACTIVE EXTRACT
    - ❖ *In-vitro* anti-oxidant activity
        - ➢ DPPH Assay
        - ➢ Hydrogen peroxide scavenging assay
    - ❖ *In-vitro* anti-inflammatory activity
6. DEVELOPMENT AND EVALUATION OF HERBAL EYE OINTMENT
7. PHARMACOLOGICAL STUDIES
    - ❖ Acute irritancy study
    - ❖ Pharmacological evaluation of formulated herbal ointment

7.PHARMACOGNOSTICAL STUDIES

7.1 MATERIAL AND METHODS

COLLECTION OF PLANT

The fresh healthy leaves of *Justicia procumabens* Linn., was collected in the month of June at Vellore near the Agricultural field.

AUTHENTICATION OF THE PLANT

The plant was authenticated by **Dr. P. Jayaraman, Ph.D, Director**, Plant Anatomy Research Centre, Tambaram (PARC/ 2016/3274)

MACROSCOPIC EVALUATION

Various organoleptic characters like colour, odour, taste and nature like size, shape, surface and thickness were observed.

MICROSCOPIC EVALUATION [31]

Fixation of leaves

The leaves were cut and fixed in FAA solution (Formalin 5ml + Acetic acid 5ml+ 90ml of 70% Ethanol). The specimens were dehydrated after 24 hours of fixing. The leaves were graded with series of tertiary butyl alcohol, as per the standard procedure.

It was carried out by gradual addition of 58 – 60º C of melting point paraffin wax until Tertiary butyl alcohol (TBA) solution attained super saturation. The specimens were cast into paraffin blocks.

The paraffin embedded specimens were sectioned with the help of Rotary Microtome. The thickness of the sections was 10 - 12µ. De-waxing of the sections was done by customary procedures. The sections were stained with haematoxylin. The stained sections were viewed under microscope.

Photomicrographs

Microscopic descriptions of tissues were supplemented with photo micrographs whenever necessary. Photo micrographs of section were taken at different magnification for focusing of different microscopical parts of the leaf.

POWDER MICROSCOPY[32,33]

The shade dried leaves were powdered and used for powder microscopic analysis. The organoleptic characters were observed and to identify the different microscopical characteristic features various staining reagents were used. Powder was stained with 1% phloroglucinol in 90% ethanol, concentrated hydrochloric acid and observed under microscope. Powder analysis is used for the detection of characteristic structures and various cell components.

HISTOCHEMICAL COLOUR REACTIONS[34,35]

The transverse sections of leaves were stained with different staining reagent such as phloroglucinol, iodine, ferric chloride, Dragendroff's reagent, toluidine blue and picric acid to observe and localize the presence of lignin contents, starch, tannins alkaloids, flavonoids and proteins as per the standard procedures. The stained sections were then washed in water to remove the excess stain and observed under a microscope.

QUANTITATIVE MICROSCOPY[36]

LENGTH AND WIDTH OF TRICHOMES

The length and width of the trichomes present in the leaves were observed under microscope. This quantitative analysis will be helpful in the identification of the drug.

The first step involved in this is calibration of the eyepiece micrometer using the stage micrometer. For determining the calibration factor, the eyepiece is removed from the microscope, then the lens is unscrewed and in the ridge the eyepieces micrometer is placed. The lens is then replaced. The stage micrometer is then placed on the stage of the microscope and focused under high power with the eyepiece coincides with each division of stage micrometer and calculate the calibration factor using the standard formula.

The stage micrometer is replaced with the slide containing the powdered drug. The slide is prepared by using the leaf powder and viewed under microscope. The width and length of trichomes are measured by focusing them on the lines of the eyepiece micrometer. Note the no. of divisions covered by the length and width of the trichomes.

STOMATAL NUMBER AND STOMATAL INDEX

The average number of stomata per square millimeter of epidermis is termed as stomatal number. The percentage proportional to ultimate divisions of the epidermis of a leaf, which has been converted into stomata, is termed as stomatal index

Procedure

Pieces of leaf between margin or midrib was cleared and mounted and the lower surface was examined by means of a microscope with a 4mm objective and an eyepiece containing a 5mm square micrometer disc. Counts were made of the numbers of the epidermal cells and of stomata within a square grid, a cell being counted if at least half of its area lies within the grid. The stomatal index was determined for both leaf surfaces.

VEIN ISLET AND VEIN TERMINATION NUMBER

The vein-islet number is the average number of vein-islets per square millimeter of a leaf surface. Vein-let termination number is defined as the number of vein-let termination per sq. mm of the leaf surface, midway between midrib of the leaf and its margin.

Procedure

Counting the number of vein-islets in an area of 4 sq. mm. of the central part of the leaf between the midrib and the margin. Cleared the piece of the leaf by boiling with chloral hydrate solution for about thirty minutes. Arranged the camera lucida and drawing board for making drawings to scale. Put stage micrometer on the microscope and using 16 mm objective, drew a line equivalent to 1 mm as seen through the microscope and constructed a square on this line. Moved the paper so that the square was seen in the eyepiece, in the centre of the field

PHYSIOCHEMICAL ANALYSIS[37,38]

The shade dried powdered leaves of *Justicia procumbens* Linn., was used for the analysis of various physiochemical parameters which is useful in the determination of quality and purity of crude drugs. Ash values, extractive values, loss on drying, foaming index, swelling index and foreign organic matters were determined as per the standard WHO guidelines which is very much useful in the determination of quality and purity of the crude drugs.

DETERMINATION OF ASH VALUES

The residue remaining after incineration is the ash content of the drug, which simply represents the inorganic salts naturally occurring in the drug or adhering to it or deliberately added to it as a form of adulteration.

TOTAL ASH

Silica crucible was heated to red hot for 30 minutes and it was allowed to cool in desiccators. About 2gm of powdered sample was weighed accurately and evenly distributed in the crucible. Dried at 100 – 105ºC for 1 hour and ignited to constant weight in a muffle furnace at 600±25ºC. The crucible was allowed to cool in a desiccator. The percentage yield of ash with reference to the air dried substance was then calculated.

Water soluble ash

The total ash was boiled for 5min with 25ml of water. The insoluble matter was then collected in an ash less filter paper. It was washed with hot water and ignited for 15min at a temperature not exceeding 450ºC. The weight of the insoluble matter was subtracted from the weight of the ash and the difference in weight represented the water soluble ash, the percentage of water soluble ash with reference to the air dried substances was calculated with reference to the air dried material.

Acid insoluble ash

Acid insoluble ash is the residue obtained after boiling the total ash with dilute hydrochloric acid, and igniting the remaining insoluble matter. This measures the amount of silica present, especially as sand and siliceous earth

Procedure

To the crucible containing total ash of the sample, 25 ml of dilute hydrochloric acid was added. The insoluble matter is collected on an ashless filter paper (Whatman 41) and washed with hot water until the filtrate is neutral. Filter paper containing the insoluble matter to the original crucible, dry on hot plate and ignite to constant weight. The residue is allowed to cool in a suitable desiccators for 30 minutes and weighed without delay. Content of acid-insoluble ash with reference to the air dried drug is calculated.

$$\text{Acid insoluble ash} = \frac{\text{Weight of the residue obtained}}{\text{Weight of the sample taken}} \times 100$$

Sulphated ash

About 3gm of air-dried substance was ignited gently at first in a crucible, until the substance was thoroughly charred. Then the residue was cooled, moistened with 1ml of sulphuric acid, heated gently until the white fumes were no longer evolved and ignited at 800 ± 25ºC, until all the black particles were disappeared. The crucible was allowed to cool, a few drops of sulphuric acid was added and heated. Then it was ignited as before, cooled and weighed. The percentage of sulphated ash with reference to the air- dried substance was then calculated.

DETERMINATION OF EXTRACTIVE VALUES

This method is used to determine the amount of active constituents in a given amount of plant material when extracted with solvents. Extractive values are useful for the evaluation of phytoconstituents especially when the constituents of a drug cannot be readily estimated by any other means. Further these values indicate the nature of the active constituents present in a crude drug.

Determination of water soluble extractive

About 5gm of the powder was weighed and macerated with 100ml of chloroform water (95ml distilled water and 5ml chloroform) in a closed flask for 24 hours. It was shaken frequently for six hours and allowed to stand for eighteen hours. It was then filtered rapidly, taking precautions against loss of solvent and 25ml of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish. 2 ml of alcohol was added to the residue

and it was dried at 105ºC for 1 hour in the hot air oven and cooled in desiccators for 30min and weighed. The process was repeated till a constant weight was obtained; the percentage of water soluble extractive value with reference to the air dried drug was calculated.

Determination of alcohol soluble extractive

The alcohol soluble extractive value is also indicative for the same purpose as water soluble extractive value. The solvent strength of alcohol varies from 20- 90%v/v. The solvent strength has to be chosen depending upon the strength of alcohol used for the extraction of powdered drug.

Procedure

About 5gm of the powder was weighed and macerated with 100ml 90% ethanol in a closed flask for 24 hours. It was shaken frequently for six hours and allowed to stand for eighteen hours. It was then filtered rapidly, taking precautions against loss of solvent and 25ml of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish. It was dried at 105ºC for 1hour in a hot air oven. The dish was cooled in desiccator and weighed. The process was repeated till the constant weight was obtained. The percentage of alcohol soluble extractive value with reference to the air dried drug was calculated.

Determination of ether soluble extractive

About 2gm of powdered drug was accurately weighed and extracted with anhydrous diethyl ether in a continuous extractive apparatus for 20hours. The ether solution was transferred to tarred porcelain dish and evaporated spontaneously. Then it was dried over phosphorous pentoxide for 18hours and the total ether extract was weighed. The extract was heated gradually and dried at 105ºC to constant weight. The loss in weight represents the volatile portion of the extract.

LOSS ON DRYING

Accurately weighed quantity of the substances was taken in a previously ignited and cooled silica crucible and the substance was evenly distributed by gentle side wise shaking. The crucible with the contents was weighed accurately. The loaded crucible and the lid were placed in the drying chamber (105ºC). The substance was heated for a specified period of time to a constant weight. The crucible was covered with the lid and allowed to cool in a desiccator at room temperature before weighing. Finally the crucible was weighed to calculate the loss on drying with reference to the air dried substance.

DETERMINATION OF FOAMING INDEX

1gm of the coarsely powdered drug was weighed and transferred to 500ml conical flask containing 100ml boiling water. The flask was maintained at temperature 80-90°C for about 30min. It was then cooled and filtered into a volumetric flask and sufficient water was added through the filtrate to make up the volume to 100ml. The decoction was poured into 10 stopper test tube (height 16cm, diameter 16mm) in successive portions of 1ml, 2ml, 3ml, 4ml up to 10ml and the volume of the liquid in each tube was adjusted with water to 10ml. The tubes were stoppered and shaked in a length wise motion for 15 seconds, two shakes per second. Allowed to stand for 15min and the height of the foam was measured. The results are assessed as follows.

If the height of the foam in every tube is less than 1cm, the foaming index is less than 100. If a height of 1cm is measured in any tube, the volume of the plant material decoction in the tube (a) is used to determine the index. If this tube is the first or second tube

in a series, prepare an intermediate dilution in a similar manner to obtain a more precise result.

If the height of the foam is more than 1cm in every tube, the foaming index is over 1000. In this case repeat the determination using a new series of dilution of the decoction in order to obtain a result. Calculate the foaming index using the following formula:

Foaming index = 1000/a

Where, a = the volume in ml of the decoction used for preparing the dilution in the tube where foaming to a height of 1cm is observed.

DETERMINATION OF SWELLING INDEX

The swelling index is the volume in ml occupied by the swelling of 1gm of plant material under specified conditions.

Procedure

A specified quantity of the plant material was accurately weighed and transferred into a 25ml glass stoppered measuring cylinder. The internal diameter of the cylinder should be about 16mm, the length of the graduated portion about 125mm, marked in 0.2ml divisions from 0 to 25ml in an upward direction. Unless otherwise indicated in the test procedure, add 25ml of water and shake the mixture thoroughly every 10min for 1hour, allowed to stand for 3 hours at room temperature. The volume in ml occupied by the plant material was measured including any sticky mucilage. Calculate the mean value of the individual determination, related to 1gm of plant material.

FOREIGN ORGANIC MATTER

The weighed quantity of leaf powder was examined for the determination of foreign organic matter by inspection with the use of lens and calculated.

QUALITATIVE AND QUANTITATIVE ESTIMATION OF HEAVY METALS AND INORGANIC ELEMENTS [40]

Plant minerals play a vital role in metabolism. Presence of elements vary with the soil, climate conditions etc. There are essential and non essential elements which may be beneficial or harmful to living things. Non essential elements like lead, arsenic, cyanide, chromium, cadmium, aluminium, silver bring about toxic effects resulting in intoxification. Hence, qualitative and quantitative estimation of inorganic elements in the plant were carried out.

QUALITATIVE ANALYSIS OF INORGANIC ELEMENTS AND HEAVY METALS

To the ash of the drug material 50%v/v hydrochloric acid was added and kept for 1 hour. It was filtered and the filtrate was used for the following tests.

Aluminium: White gelatinous precipitate of aluminium hydroxide is formed on addition of ammonia solution. It is slightly soluble in excess of the reagent. The precipitate dissolves readily in strong acid and base, but after boiling it becomes insoluble.

Arsenic: Arsenious salts in neutral solution react with solution of copper sulphate to form green precipitate (scheele's green) which on boiling gives a red precipitate of cupric oxide.

Borate: The mixture obtained by the addition of sulpuric acid and alcohol (95%) to a borate when ignited, burns with flame tinged with green.

Calcium: Solution of calcium salts, when treated with ammonium carbonate solution, yield a white precipitate after boiling and cooling the mixture (it is insoluble in ammonium chloride solution).

Carbonate: Carbonate, when treated with dilute acid effervescence, liberating carbon dioxide which is colourless and produces a white precipitate in calcium hydroxide solution.

Chlorides: Chlorides, when treated with silver nitrate solution, yield a white crude precipitate which is insoluble in nitric acid, but soluble after being well washed with water, in diluted ammonia, from which it is re precipitated by the addition of nitric acid.

Copper: An excess of ammonia, added to a solution of a cupric salt, produces first a bluish precipitate and then a deep blue coloured solution.

Iron: Solution of ferric salts, when treated with potassium ferrocyanide solution, yields an intense blue precipitate which is insoluble in dilute HCL.

Lead: Strong solution of lead salts, when treated with HCL, yield a white precipitate. Which is soluble in boiling water and is re deposited as crystals when the solution is cooled.

Magnesium: Solution of magnesium salts, when treated with ammonium carbonate solution and boiled, yield a white precipitate, but yield no precipitate in the presence of ammonium chloride solution.

Mercury: Solution of mercury salts, when treated with sodium hydroxide solution, yields a yellow precipitate.

Nitrate: With solution of ferrous sulphate no brown colour was observed but if sulphuric acid is added (slow from the side of the test tube), a brown colour is produced at the junction of two liquids, indicating the presence of nitrates.

Phosphate: Solution of phosphate when treated with silver nitrate with dilute ammonia solution and in dilute nitric acid yield yellow precipitate of normal silver ortho phosphate (distinction from meta and pyrophosphate) solution.

Potassium: Moderately strong potassium salts, which have been previously ignited to remove ammonium salts, when treated with perchloric acid (60%) yield a white crystalline precipitate.

Silver: Solution of silver salts, when treated with potassium iodide solution yield a cream coloured precipitate which is insoluble in dilute ammonia solution and in nitric acid.

Sulphates: Solution of sulphates, when treated with lead acetate solution yields a white precipitate which is insoluble in ammonium acetate solution and in sodium hydroxide.

7.2 RESULTS AND DISCUSSION

The results of pharmacognostical studies are as follows,

MACROSCOPICAL STUDIES

Organoleptic evaluation

Colour - Green

Taste - Bitter

Odour - Odourless

Texture - Smooth

MACROSCOPY

Type – Trifoliate, Alternate, Compound

Shape – Oblong, oval

Margin – Entire

Apex – Acuminate

Base – Obtuse

Size – 1-3cm in length

1-2 cm in breadth

Surface – Upper – Smooth dark green

Lower – Smooth pale green

Texture – Fine

Venation – Reticulate

Leaflets – Triangular medium

Fig.3 Morphology of leaf

MICROSCOPY OF LEAF

Transverse section of the leaf shows epidermis, mesophyll and vascular bundle. Leaf has prominent lateral vein and midrib with uniformly thin lamina prominent conical adaxial cone and semicircular abaxial part.

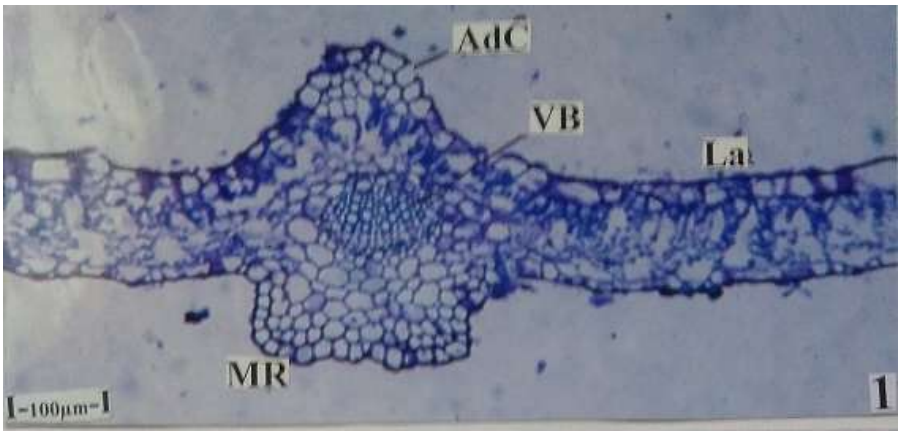

Fig. 4 T.S of leaf through midrib

La-Lamina, VB- vascular bundle, AdC-Adaxial cone, MR-Midrib,

Midrib has slightly broad adaxial hump and flat rectangular abaxial part. Epidermis is made up of squarish cells with thin cell walls. A small group of 2 to 3 layers of collenchyma cells are present in midrib zone just beneath the epidermis. Palisade parenchyma cells are horizontally transcurrent across the adaxial part. Abaxial midrib has prominent layer of epidermis with thick walls and prominent cuticle.

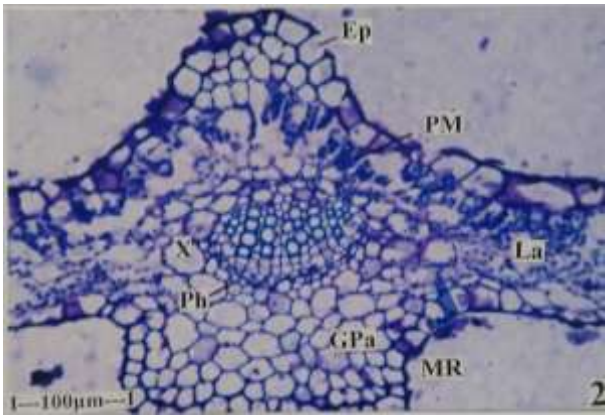

Fig 5: T.S of Midrib – enlarged

Ep- Epidermis, PM-Palisade Mesophyll, Ph-Phloem, X-Xylem, La-Lamina, GPa-Ground Parenchyma.

Mesophyll made up of thin walled, compactly arranged parenchyma. Lateral vein is 350 mm in vertical plane and 200 mm in horizontal plane. Midrib is 450 mm and 350 mm horizontally. Vascular bundle of the lateral vein is single top-shaped and collateral. The vascular bundle of the midrib is slightly broader and bundle sheatisabsent.

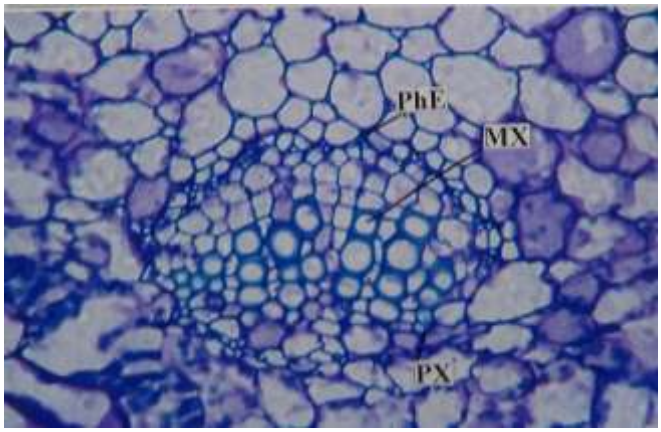

Fig 6 : T.S of Midrib with vacular bundle

MX-Meta Xylem, PhE-Phloem elements, PX-protoxylem

Lamina is 150 mm thick it has thick adaxial epidermis with large squarish cells which are 30-40 mm thick. Abaxial epidermis is thin walled and stomatiferous. Mesophyll consists of single layer of thin walled, loosely arranged palisade parenchyma and 4 to 5 layers of spongy parenchyma with wide air cavities (Fig. 3.). Palisade zone is 80 mm in length.

Epidermal trichomes

Two types of trichomes are present on the lamina and veins.

Nonglandular or covering trichomes:

This type of trichomes are more abundant. It is two or three celled, uniseriate, unbranched and thin walled pointed tip. Trichome may be straight or curved. Basal epidermal cell of trichome is dilated. Trichome is about 220 mm long.

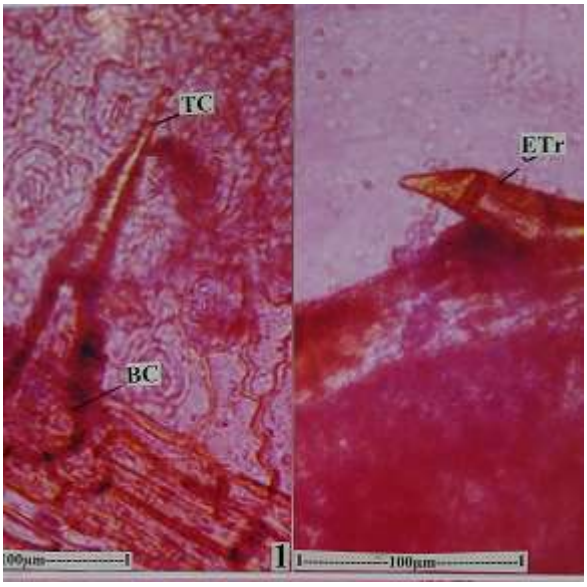

Fig 7: Nonglandular trichomes from the submarginal part of the lamina

BC- Basal cell, ETr- Epidermal Trichome, TC- Terminal cell

Glandular trichomes:

These are two types. One type of trichome is small spherical type situated in shallow cavity of the epidermis, it has a short stalk and globular multicellular body with dense content. Second type of trichome has long, unicellular, uniformly thick short stalk cell and a crown of

rosette cells. This type is less frequent and occurs mostly on the lower epidermis. Trichome is 450 □m long, terminal rosette of cells 30 □m wide.

Stomata:

Stomata occur only in lower epidermis of lamina and on young stem. Stomata slightly raised above the epidermal level. Stomata are diacytic. Most of the stomata have more than one circle of subsidiary cells. The stomata are 11 □m^2. The guard cells are 90 x 50 mm size.

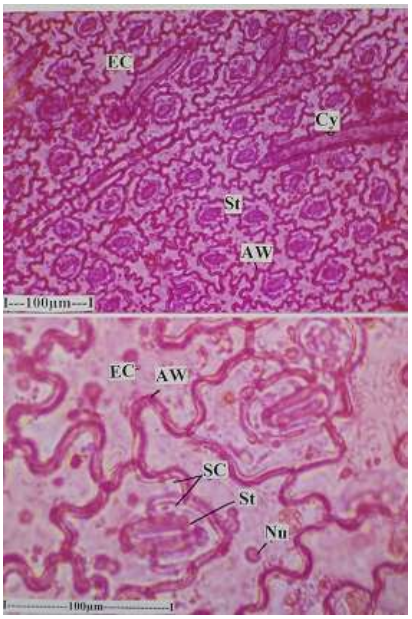

Fig 8 : Epidermal peeling of the leaf showing stomata

EC - Epidermal cells, AW–Anticlinal Walls, St - Stomata, Sc - Subsidiary cells, Nu - Nucleus

Cy-Cystoliths

POWDER MICROSCOPY

ORGANOLEPTIC CHARACTERS

Nature	:	Coarse powder
Colour	:	Light green colour
Odour	:	Characteristic odour
Taste	:	Bitter taste

The powdered leaves of *Justicia procumbens* under microscopic investigation showed the presence of lignified fibres, xylem, phloem fibres, parenchymatous cells and calcium carbonate crystals (cystoliths), covering trichomes, glandular trichomes and diacytic stomata.

Fibres

Fibres are lignified and scattered.

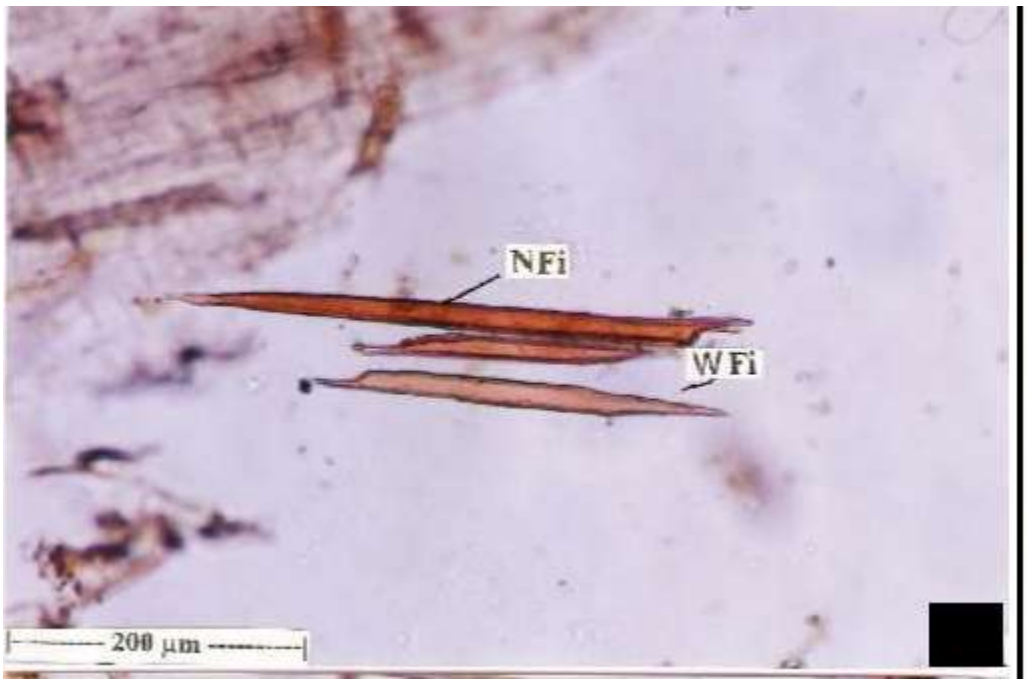

Fig9. Fibres

NFi- Narrow fibres, WFi- wide fibres

Phloem fibres

Phloem fibres occur lengthwise in groups of 3-5 cells, the brown masses are adhering to the fibres.

Cystolith*s:*

Calcium carbonate cystoliths are abundant in the epidermal cells of leaf and young stem. Cystoliths are long and cylindrical with warty surface. They occur in the enlarged epidermal cells called lithocysts.

Cystoliths are 220 □m long and 20 □m width .

Fig 10. Cystolith isolated from the leaf

Cy- Cystolith

HISTOCHEMICAL COLOUR REACTIONS

Transverse section of leaves of *Justicia procumbens* Linn., was treated with various reagents like Dragendroff's reagent, N/50 iodine, phloroglucinol and Conc.HCl, ferric chloride, picric acid and toludine blue. The histochemical color reactions of transverse section of leaves showed different stained cells were observed.

Table :2 Histochemical colour reactions of *Justicia procumbens* Linn.,

S. No	Chemicals	Test for	Nature of change	Degree of	Location
1	**Phloroglucinol + HCl**	Lignin	Magneta	+	Xylem
2	**N / 50 Iodine solution**	Starch	Blue	+	Mesophyll
3	**Dil. Ferric chloride**	Tannin	Black	+	Bundle cap
4	**Dragendorff's Reagent**	Alkaloid	Brown	+	Xylem
5	**Picric acid**	Protein	Intense	+	Bundle cap
6	**Toluidine blue**	Flavonoids	Bluish green	+	Inter cellular zones and xylem

Note: + - Indicates the presence and absence

The histochemical analysis on the leaves of *Justicia procumbens* Linn., showed the presence of alkaloids, starch grains, lignin, flavonoids and tannins.

QUANTITATIVE MICROSCOPY

Linear measurement of trichomes

The length and width of the trichomes were measured in the powdered leaves of *Justicia procumbens* Linn., and the results were shown in table no:

TABLE : 3 LINEAR MEASUREMENT OF TRICHOMES

Trichomes type	Length(µm)	Width (µm)
Non-glandular	83.33	19.7
Glandular	64.04	14.97

***Data presented in the table are averages of 20 observations**

Stomatal number and stomatal index

Stomata were measured in the powdered leaves of *Justicia procumbens* Linn., and the results were shown in table no: 4.

TABLE : 4 Stomatal number and stomatal index

Leaf surface	Stomata Type	Stomatal number(per sq.mm)	Stomatal index%
Upper	Diacytic	4-5	2.13
Lower	Diacytic	8-10	8.35

***Data presented in the table are averages of 20 observations**

VEIN-ISLET, VEIN TERMINATION NUMBER AND PALISADE RATIO

DETERMINED	VALUE (per sq.mm)
Vein islet number	**12-16**
Vein termination number	**15-20**

PHYSIOCHEMICAL CONSTANTS

Physiochemical constants like total ash values, acid insoluble ash, water soluble ash, extractive values, loss on drying, swelling index, foaming index, foreign organic matter were studied.

TABLE 5: PHYSIOCHEMICAL ANALYSIS OF THE LEAVES OF *Justicia procumbens* Linn.,

S.NO	PHYSIO-CHEMICAL CONSTANTS	RESULTS (%W/W)
I	**ASH VALUE**	
1.	Total ash	15.50±0.16
2.	Water soluble ash	5.26±0.75
3.	Acid insoluble ash	2.69±0.33
4.	Sulphated ash	10.68±0.36
II	**EXTRACTIVE VALUE**	
1.	Water soluble extractive	3.56±0.42
2.	Alcohol soluble extractive	6.89±0.53
3.	Ether soluble extractive	5.63±0.21
4.	Non-volatile ether soluble extractive	4.64±0.52
III	**Loss on drying**	1.55±0.50
IV	**Foaming index**	Nil
V	**Swelling index**	Nil
VI	**Foreign organic matter**	Nil

Values are expressed as Mean ± SD, n=3

The total ash, acid insoluble ash, water soluble ash and sulphated ash were found to be 15.50±0.16% w/w, 2.69±0.33% w/w, 5.26±0.75% w/w and 10.68±0.36% w/w respectively. The water soluble extractive, alcohol soluble extractive, ether soluble extractive were found to be 3.56±0.42% w/w, 6.89±0.53% w/w, 5.63±0.21% w/w, respectively. Loss on drying was found to be 1.88±0.50% w/w. The foaming index and swelling index was found to be nil this indicates the absence of mucilage and saponins

INORGANIC ELEMENTS AND HEAVY METAL ANALYSIS

Qualitative and quantitative estimation of inorganic metals were analysed the results were tabulated as follows.

Table No 6. Qualitative estimation of inorganic elements of *Justicia procumbens*

S.No	INORGANIC ELEMENTS	OBSERVATIONS
1.	Aluminium	+
2.	Chloride	+
3.	Copper	-
4.	Calcium	+
5.	Iron	-
6.	Borate	+
7.	Potassium	+
8.	Carbonate	-

Table No 7: Quantitative estimation of inorganic elements of *Justcia procumbens*

S.NO	INORGANIC ELEMENTS	TOTAL AMOUNT (% W/W)
1.	Aluminium	0.018
2.	Chloride	0.044
3	Calcium	0.032
4.	Borate	0.004
5.	Potassium	0.049
6.	Nitrate	0.011

Quantitative estimation of Heavy metals by ICP OES method

The quantification of the individual heavy metals was analyzed for the powdered mixture of *Citrus maxima* by ICP-OES technique the following metals like arsenic, lead, cadmium were detected and quantified, results are given in the following table

Table No 8: Quantitative estimation of Heavy metals

S.No	Element	Results (ppm)	Specification (as per WHO)
1.	Mercury	Not detected	Not more than 0.5ppm
2.	Arsenic	0.023	Not more than 5.0ppm
3.	Lead	0.032	Not more than 10ppm
4.	Cadmium	0.001	Not more than 0.3ppm

The above observation showed that the heavy metals are within the limits as per WHO standard and it is safe to consume internally.

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8. PHYTOCHEMICAL STUDIES

8.1.MATERIALS AND METHODS

Phytochemical evaluation is used to determine the nature of phytoconstituents present in the plant by using suitable chemical and analytical techniques. Phytochemical analysis is very much important because the therapeutic activity of the plant is based on the constituents present in the drug. Therefore a complete investigation is required to characterize the Phytoconstituents qualitatively and quantitatively.

PREPARATION OF EXTRACTS

Fresh healthy leaves were collected, dried in shade, coarsely powdered and successively extracted with solvents of increasing polarity like n-hexane, ethyl acetate and ethanol by continuous percolation process using soxhlet apparatus. After extraction each extracts were concentrated by using rotary vacuum evaporator. It is dried and the percentage yield was calculated. Appearance and consistency of the extracts were also noted.

PRELIMINARY PHYTOCHEMICAL SCREENING[38,39]

The leaf powder and extracts were subjected to qualitative chemical analysis for the identification of active constituents in each extracts and the powdered leaves.

DETECTION OF ALKALOIDS

Dragendorff's test

To the sample 5ml of 2M HCl was added. Then 1ml of Dragendorff's reagent was added and examined for an immediate formation of an orange red precipitate.

Mayer's test

To the substance little quantity of dilute hydrochloric acid and Mayer's reagent were added and examined for the formation of white precipitate.

Wagner's test

The test substance was treated with little amount of Wagner's reagent and examined for the formation of reddish brown precipitate.

DETECTION OF GLYCOSIDES

Borntrager's test

The powdered material was boiled with 1ml of sulphuric acid in a test tube for five minutes. Filtered while hot, cooled and shaken with equal volume of chloroform. The lower layer of solvent was separated and shaken with half of its volume of dilute ammonia. A rose pink to red colour is produced in the ammonical layer indicates the presence of anthroquinone glycosides.

Modified Borntrager's test

The test material was boiled with 2ml of the dilute sulphuric acid. This was treated with 2ml of 5% aqueous ferric chloride solution (freshly prepared) for 5 minutes, and shaken with equal volume of chloroform. The lower layer of solvent was separated and shaken with half of its volume of dilute ammonia. A rose pink to red colour is produced in the ammonical layer.

Legal's test

The test sample when treated with sodium nitropruside in pyridine and methanolic alkali. Formation of a pink red colour indicates the presence of cardiac glycosides.

DETECTION OF STEROIDS AND TRITERPENOIDS

Libermann Burchard's Test

The powdered drug was treated with few drops of acetic anhydride, boiled and cooled. Conc.sulphuric acid was added from the sides of the test tube formation of brown ring is formed at the junction of two layers and upper layer turns green which shows presence of steroids and formation of deep red color indicates presence of tri terpenoids.

Salkowski Test

The extract was treated with few drops of concentrated sulphuric acid, red color at lower layer indicates presence of steroids and formation of yellow colored lower layer indicates presence of tri terpenoids.

DETECTION OF FLAVONOIDS

Shinoda's test

Small quantity of extract was dissolved in alcohol, to this pieces to magnesium followed by concentrated hydrochloric acid were added drop wise and heated. Appearance of magenta colour shows the presence of flavonoids.

Alkaline reagent test

Small quantity of the extract was dissolved in aqueous sodium hydroxide and appearance of yellow colour indicates the presence of flavonoids.

DETECTION OF CARBOHYDRATES

Molisch's test

To the test solution few drops of alcoholic alpha napthol and few drops of conc. sulphuric acid were added through the sides of test tube, purple to violet color ring appears at the junction indicates the presence of carbohydrates.

Fehling's test

The test solution was mixed with Fehling's I and II, heated and examined for the appearance of red coloration for the presence of sugar.

DETECTION OF TANNINS

Lead acetate test

The test solution was mixed with basic lead acetate solution and examined for formation of a white precipitate.

Ferric chloride test

A few drops of 5% aqueous ferric chloride solution was added to 2ml of an aqueous extract of the drug and examined for the appearance of bluish black color.

DETECTION OF PROTEINS

Biuret test

The sample was treated with 5-8 drops of 10% w/w copper sulphate and sodium hydroxide solution and observed for the presence of violet colour.

DETECTION OF SAPONINS

A drop of sodium bicarbonate solution was added to the sample and the mixture was shaken vigorously and left for 3 minutes. Development of any honey comb like froth was examined.

DETECTION OF GUMS AND MUCILAGE

The small quantities of test substance were dissolved in 5 to 10ml of acetic anhydride by means of heat, cooled and added 0.05ml of conc. sulphuric acid. Formation of bright purplish red color indicates the presence of gums.

Sample was treated with ruthenium red, formation of red colour indicates the presence of mucilage.

DETECTION OF FIXED OILS AND FATS

Small quantities of extracts were pressed between two filter papers. An oily stain on the filter paper indicates the presence of fixed oils and fats.

QUANTITATIVE ESTIMATION OF TOTAL PHENOLIC AND TOTAL FLAVONOID CONTENT [46,47]

Total phenolic content (Folin – Ciocalteu's assay)

Total phenolic content of the extracts were determined using Folin – Ciocalteu's assay. 0.5ml extract solutions were mixed with 2.5ml of 10 fold diluted Folin Ciocalteu's reagent and 2.5ml of 7.5% sodium carbonate. After incubation at 40°C for 30 minutes, the absorbance of the reaction mixtures was measured at 765nm in a spectrophotometer. Three replicates were made for each test sample. Gallic acid was used as a standard and total phenolic content of the extract was expressed in mg of Gallic acid equivalents (mg GAE/g extract).

Total Flavanoid content

Total flavanoid content was determined by calorimetric method, using quercetin as a standard. The test samples were individually dissolved in DMSO. Then the sample solution (150 µl) was mixed with 150 µl of 2% aluminium chloride. After 10min of incubation at ambient temperature, the absorbance of the supernatant was measured at 435nm using spectrophotometer. Three replicates were made for each test sample. The total flavanoid content was expressed as quercetin equivalent in mg/gm extract (mg QRT/gm extract).

FLUORESCENCE ANALYSIS [44]

Many crude drugs shows Fluorescence when the sample is exposed to UV radiation. Evaluation of crude drugs based on fluorescence in day light is not much used, as it is usually unreliable due to the weakness of the fluorescent effect. Fluorescent lamps are fitted with suitable filter, which eliminate visible radiation from the lamp and transmits UV radiation of definite wavelength was used for the study several crude drugs show

characteristic fluorescence which is very much useful for their evaluation.

Fluorescence analysis for the extracts and the powdered drug were carried out with various reagents to identify the presence of chromophores. The importance of fluorescence analysis is that UV light shows the fluorescent nature of the compound whereas fluorescence cannot be observed in day light. Hence it is performed according to the standard procedures.

CHROMATOGRAPHY[49,50]

Chromatographic methods are important analytical tool for the separation and identification of active components present in the plant.

THIN LAYER CHROMATOGRAPHY

Principle

The principle of separation is adsorption. It is reliable technique in which solute undergoes distribution between two phases, stationary and mobile phase. The mobile phase flows through because of capillary action (against gravitational force). The compounds having higher affinities towards the stationary phase eluted slower were as the compound having lesser affinities towards stationary phase eluted faster.

Selection of mobile phase

Solvent mixture was selected on the basis of the phytoconstituents present in each extract. Factors such as nature of components, stationary phase, polarity, influence the rate of separation of constituents was considered. From the vast analysis, best solvents were selected which showed good separation with maximum number of components.

$$R_f \text{ values} = \frac{\text{Distance travelled by solute from the baseline}}{\text{Distance travelled by solvent from the baseline}}$$

HPTLC- FINGERPRINT PROFILE

HPTLC is one of the versatile chromatographic method which helps in the identification of compounds and thereby authentication of purity of herbal drugs. The time required in this method for the demonstration of most of the characteristic constituents of a drug is very quick and short. In addition to qualitative detection, HPTLC also provides semi- quantitative information on major active constituents of a drug, thus enabling an assessment of drug quality.

HPTLC serves as a convenient tool for finding the distribution pattern of phytoconstituents which is unique to each plant. The fingerprint obtained was suitable for monitoring the identity and purity of drugs and for the detection of adulteration and substitution. HPTLC technique is helpful in order to check the identity, purity and to standardize the quantity of active principles in the herbal extract.

Instrument Conditions:

Sample used	**:**	Ethanolic Extract of leaves of *Justicia procumbens*
Instrument	**:**	CAMAG HPTLC
Stationary phase	**:**	Aluminum coated silica Gel-Merk F254
Volume of injection	**:**	20µl
Mobile phase	**:**	Ethyl acetate : Toluene : Methanol : Formic acid (6:2:1:1)
Derivatizing agent	**:**	10% Anisaldehyde in sulphuric acid
Lamp	**:**	Tungsten
Lambda max	**:**	400-800nm

The estimation has been done using the following chromatographic conditions. Chromatography was performed on a 12 × 3 cm (H x W) pre-activated HPTLC silica Gel Merk F254 plate. Samples were applied to the plate as 6mm wide band with an automatic TLC applicator 8mm from the bottom.

8.2 RESULTS AND DISCUSSION

Phytochemical investigations were carried and the results are as follows.

Table no 9 : Percentage yield of successive extracts of leaves of *Justicia procumbens* Linn.,

S.NO	EXTRACT	METHOD OF EXTRACTION	PHYSICAL NATURE	COLOUR	YIELD (%W/W)
1.	**n-Hexane**	Continuous percolation method using Soxhlet apparatus	Semisolid	Green colour	1.70
3.	**Ethyl acetate**		Solid	Dark Green	1.10
4.	**Ethanol**		Semisolid	Green colour	2.30

QUALITATIVE PHYTOCHEMICAL ANALYSIS

TABLE NO 10 : PRELIMINARY PHYTOCHEMICAL ANALYSIS

S.NO	CHEMICAL CONSTITUENTS	POWDERED DRUG	n-HEXANE EXTRACT	ETHYL ACETATE EXTRACT	ETHANOL EXTRACT
1.	**Steroids**	+	+	+	+
2.	**Glycosides**	-	-	-	-
3.	**Saponins**	-	-	-	-
4.	**Flavonoids**	+	-	+	+
5.	**Tannins**	+	-	+	+
6.	**Triterpenes**	+	+	-	-
7.	**Proteins**	+	-	-	+
8.	**Alkaloids**	+	-	+	+
9	**Carbohydrates**	+	-	+	+
10.	**Fats and oils**	-	-	-	-

Note: + - Indicates the presence and absence

From the qualitative analysis, it was observed that the ethanol extract showed the presence of maximum active constituents such as flavonoids, tannins, triterpenes, proteins and alkaloids. All these compounds were found in the powdered leaf also. The ethyl acetate extract also showed the presence of more number of active constituents. n-Hexane extract showed the presence of steroids and teriterpenes.

TOTAL FLAVONOID CONTENT

Total flavonoid content was determined and compared with that of standard and thus ethanolic extract was found to have higher absorbance than ethyl acetate. It is represented in table

Table No 11 : Determination of Total Flavanoid Content

S.No	Concentration of standard solution(µg/ml)	Absorbance(765nm)
1.	2	0.07
2.	4	0.10
3.	6	0.13
4.	8	0.16
5.	10	0.20
6.	Ethyl Acetate	0.12
7.	Ethanol	0.17

Standard calibration curve for determination of Total Flavonoid Content

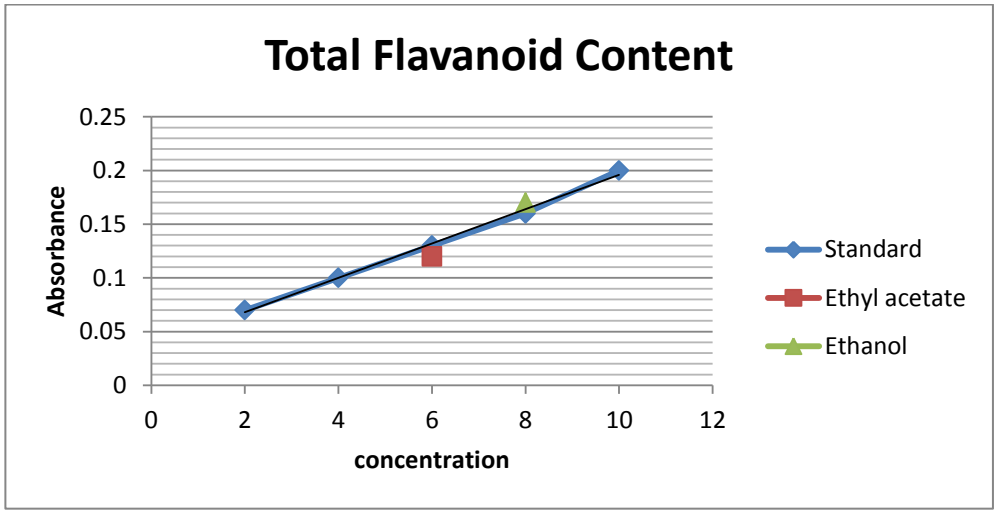


Fig 11 : Total Flavonoid content

TOTAL PHENOLIC CONTENT

Total phenolic content was determined and compared with that of standard and thus ethanolic extract was found to have higher absorbance than ethyl acetate. It is shown in Table

Table 12: Determination of Total Phenolic Content

S.No	Concentration of standard solution(µg/ml)	Absorbance
1.	20	0.13
2.	40	0.16
3.	60	0.19
4.	80	0.22
5.	100	0.25
6.	Ethyl Acetate	0.15
7.	Ethanol	0.17

Calibration curve for determination of total Phenolic content

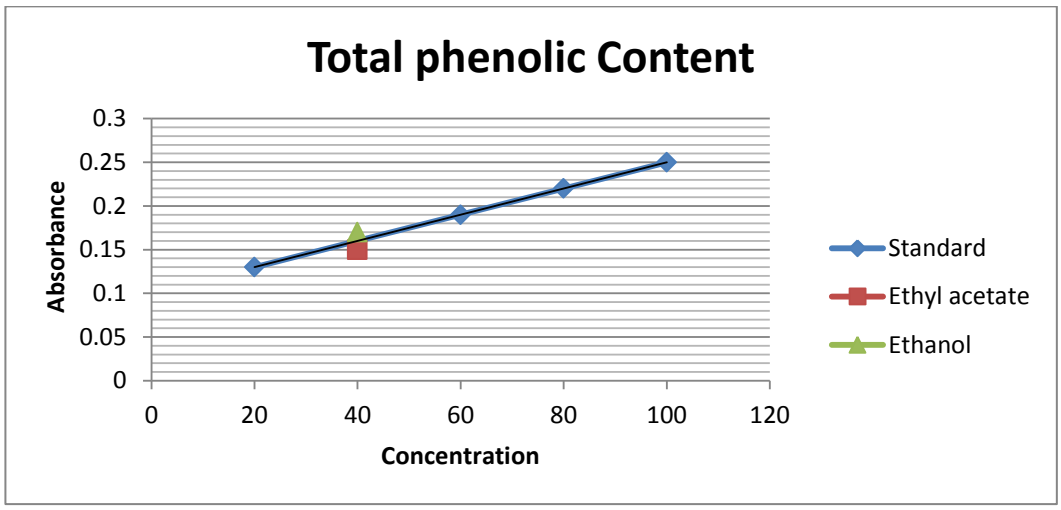


Fig12 : Total Phenolic Content

The Flavonoid and phenolic content present in the extract were determined quantitatively.

Table 13 : Quantitative estimation of phytoconstituents

S.No	EXTRACT	TOTAL FLAVANOID CONTENT	TOTAL PHENOLIC CONTENT
1.	Ethyl acetate	5.250µg/mg	50.25 µg/mg
2.	Ethanol	8.375µg/mg	70.42 µg/mg

FLUORESCENCE ANALYSIS

TABLE 14: Fluorescence characteristic of powdered leaves of *Justicia procumbens* Linn.,

S. NO	TREATMENT	DAY LIGHT	SHORT UV (254nm)	LONG UV (366nm)
1.	Powder	Pale green	Green	Brown
2.	Powder + water	Pale green	Green	Brown
3.	Powder + NaoH	Green	Yellowish green	Green
4.	Powder + HCl	Pale Green	Pale green	Black
5.	Powder + Acetic acid	Green	Green	Black
6.	Powder + Alc.NaoH	Green	Yellowish green	Green
7.	Powder + Picric acid	Yellow	Yellowish green	Yellowsih green
8.	Powder + Sulphuric acid	|Dark Green	Black	Black
9.	Powder + Nitric acid	Orange	Green	Black
10.	Powder + Iodine	Dark Green	Dark Green	Black
11.	Powder + Fecl	Dark yellowish green	Dark greyish	Black
12.	Powder + KOH	Brown	Black	Black
13.	Powder + alc.KOH	Brown	Black	Black
14.	Powder + ammonia	Light green	Green	Dark green
15.	Powder + ethanol	Light green	Green	Dark green

TABLE 15 Fluorescence analysis of various

extracts of *Jsticia procumbens* Linn.,

S.NO	TREATMENT	DAY LIGHT	SHORT UV (254nm)	LONG UV (366nm)
1.	**n-Hexane**	Green	Light green	Green
2.	**Ethyl acetate**	Dark green	Black	Black
3.	**Ethanol**	Dark green	Black	Black

There was no characteristic fluorescence were seen with either the powdered leaves or the extracts.

CHROMATOGRAPHIC STUDIES

THIN LAYER CHROMATOGRAPHY

Thin layer chromatography was done with all the three extracts and their R_f values were tabulated in the table no: 16

TABLE 16. Thin layer chromatographic studies of all three extracts

S.NO	EXTRACTS	SOLVENT SYSTEM	NO. OF SPOTS	R_f VALUES
1.	**n-Hexane**	Toluene : ethyl acetate : acetonitrile (3:6:1)	1	0.65
2.	**Ethyl acetate**		2	0.37,0.47
3.	**Ethanol**		4	0.39,0.5,0.74

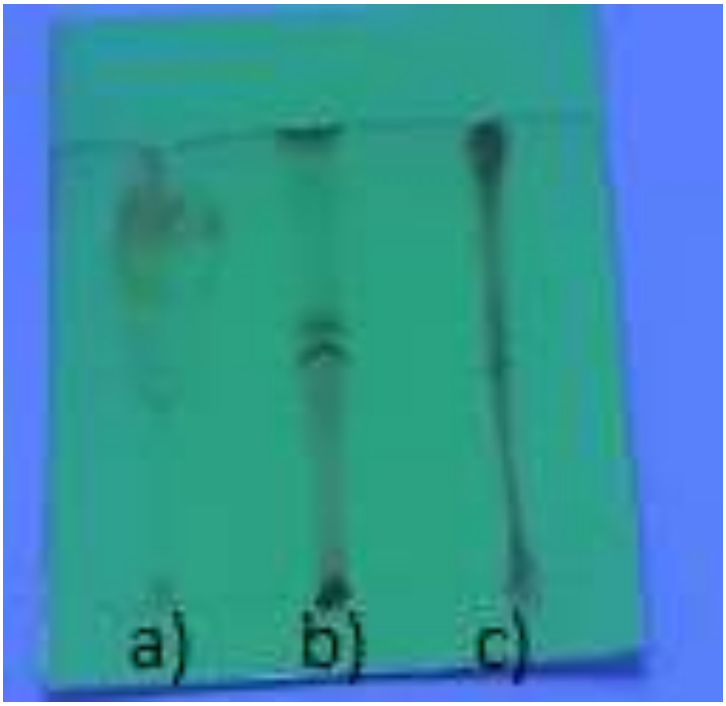


FIG .13. TLC OF THREE EXTRACTS OF LEAVES OF *Justicia procumbens* Linn

a) Hexane b) Ethanol c) Ethyl acetate

HPTLC FINGER PRINT PROFILE

HPTLC Finger print Data of Ethanolic Extract of *Justicia procumbens* Linn.,

Since more number of phytoconstituents were present in ethylacetate and ethanolic extract, they were selected for the HPTLC studies. High performance thin layer chromatography (HPTLC) finger printing was performed with the ethylacetate and ethanolic extract of *Justicia procumbens* Linn.,

FIG 14: HPTLC of Ethyl acetate extract

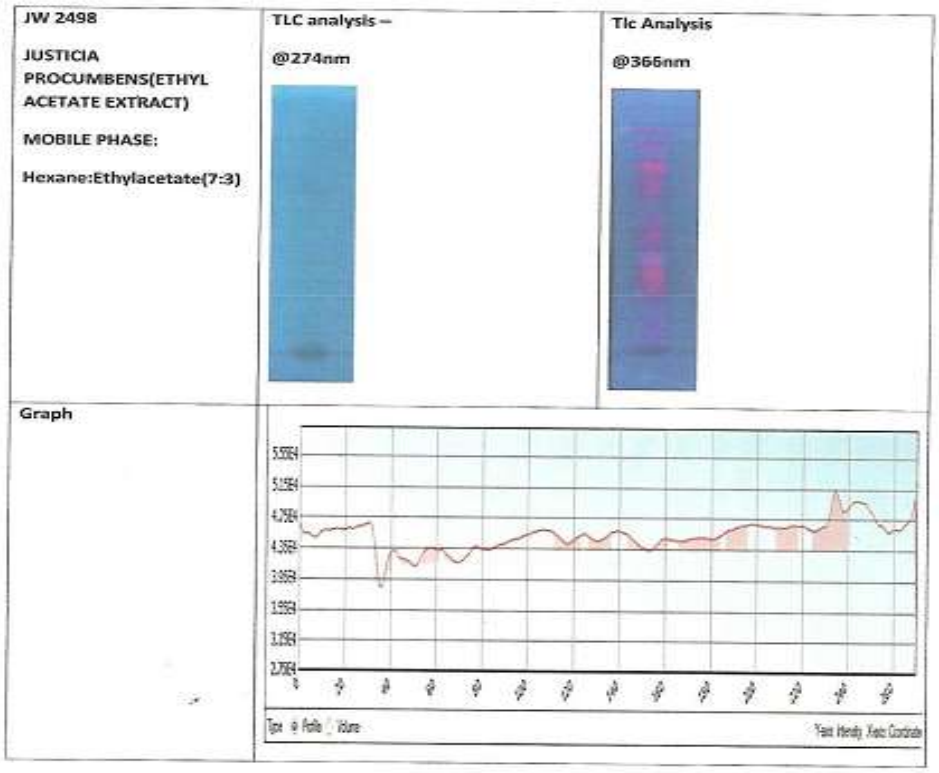


FIG 15: Histogram of ethyl acetate extract of

Leaves of *Justicia procumbens* Linn.,

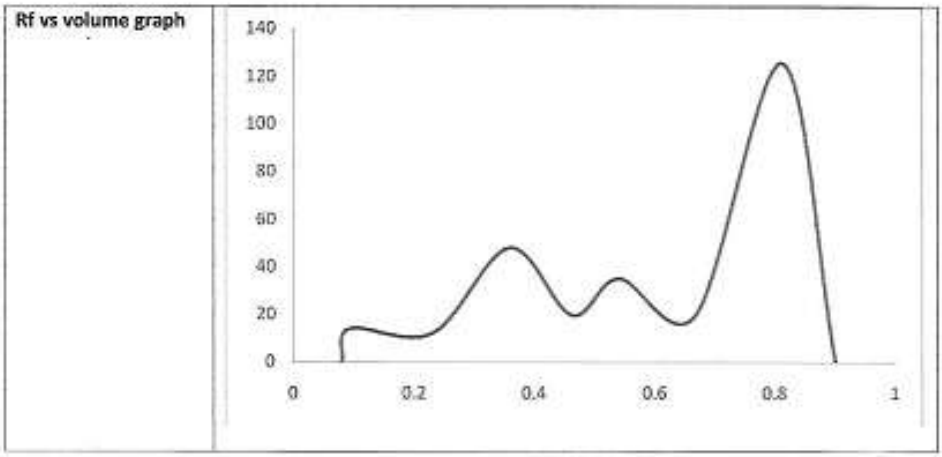


FIG 16: HPTLC finger print of ethyl acetate extract of *Justicia procumbens* Linn.,

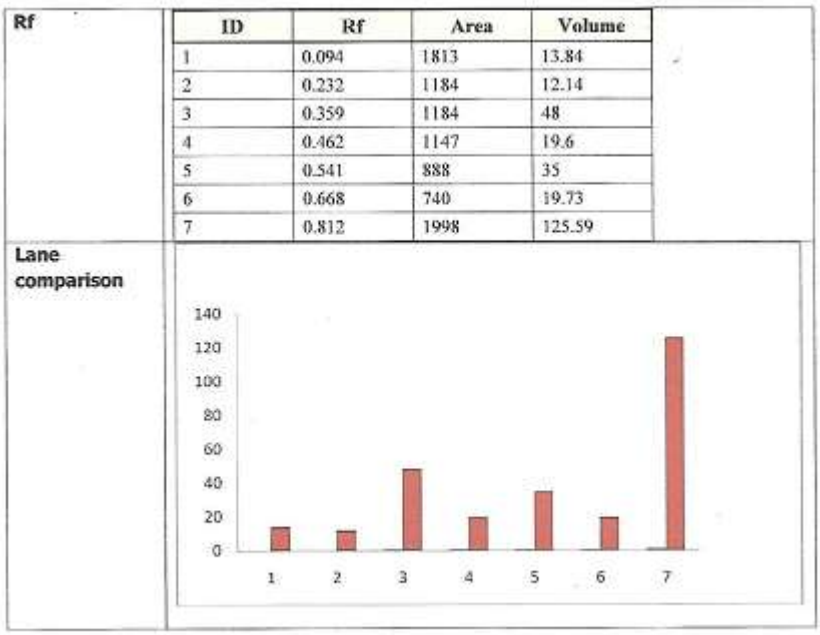


FIG. 17: HPTLC OF ETHANOLIC EXTRACT

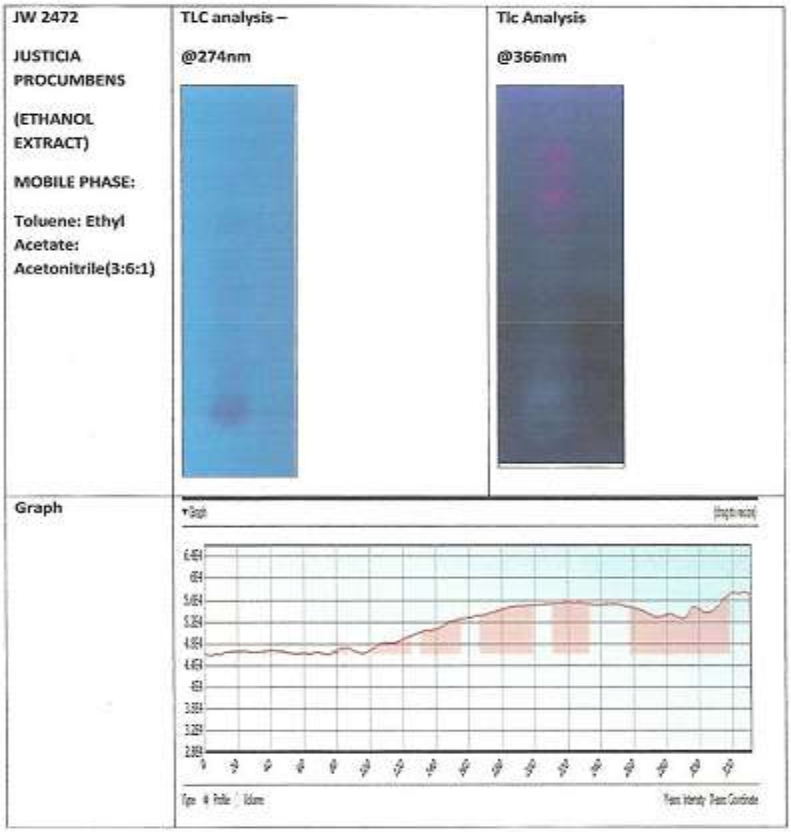


FIG 18: HISTOGRAM OF ETHANOLIC EXTRACT

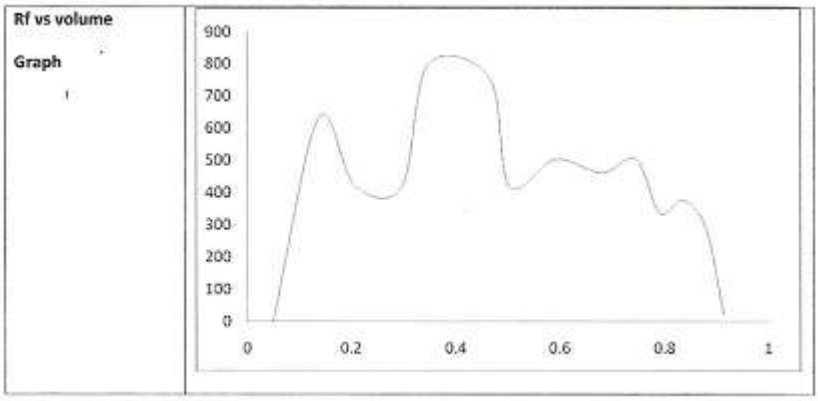
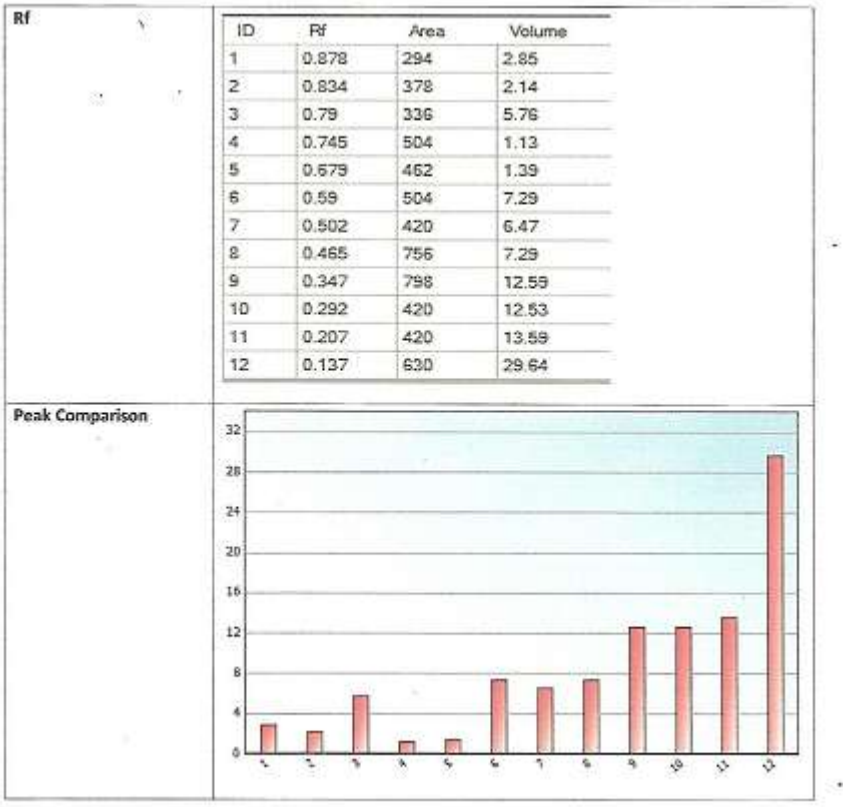


FIG 19: HPTLC FINGER PRINT OF ETHANOLIC EXTRACT OF *Justicia procumbens* Linn.,



ID	Rf	Area	Volume
1	0.878	294	2.85
2	0.834	378	2.14
3	0.79	336	5.76
4	0.745	504	1.13
5	0.679	462	1.39
6	0.59	504	7.29
7	0.502	420	6.47
8	0.465	756	7.29
9	0.347	798	12.59
10	0.292	420	12.53
11	0.207	420	13.59
12	0.137	630	29.64

The finger print of ethyl acetate and ethanolic extract showed 7 and 12 peaks respectively with different R_f values.

Since secondary metabolites are responsible for biological activity, this study would be the leading pathway of information for selection of extract for pharmacological activity and isolation of constituents responsible for the activity.

9. SELECTION OF ACTIVE EXTRACT

9.1. MATERIALS AND METHODS

The plant *Justicia procumbens* Linn., contains many active constituents in each extracts. In order to select the best extract all the extracts were subjected to *in-vitro* studies like *in vitro* antioxidant and anti-inflammatory activity. This study is used for the selection of best active extract which would take to proceed the further activities.

IN VITRO ANTIOXIDANT ACTIVITY

Antioxidant is a molecule that inhibits oxidation of other molecule which produces free radicals. These radicals in turns produce chain reactions there by cause damage to the cells, resulting in development of various ailments. Antioxidants terminate these chain reactions by removing free radicals and inhibiting oxidative reactions. Therefore, antioxidant with free radical scavenging effect will be of greater importance in the prevention and therapeutics of disease.

DPPH ASSAY (2, 2-DIPHENYL -1-PICRYLHYDRAZYL)

Antioxidants react with DPPH and reduce it to DPPH-H and as consequence the absorbance decreases. The degree of discolouration indicates the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen donating ability. Different volumes (1.25-10µ l) of plant extracts were made up to 40µ l with DMSO and 2.96ml DPPH (0.1mM) solution was added. The reaction mixture was incubated in dark condition at room temperature for 20 minutes. After 20 minutes, the absorbance of the mixture was read at 517nm. 3ml of DPPH was taken as control.

$$\% \text{ Inhibition} = \frac{\text{Abs (Control)- Abs (Test)}}{\text{Abs (Control)}} \times 100$$

***IN VITRO* ANTI-INFLAMMATORY STUDY:**

PRINCIPLE

Protein denaturation has been well correlated with the occurrence of the inflammatory response and leads to inflammatory diseases. Tissue injury might be referred to denaturation of protein constituents of cells or of intercellular substance. Hence, the ability of a substance to inhibit the denaturation of protein signifies apparent potential for anti-inflammatory activity.

EGG ALBUMIN DENATURATION METHOD:

The reaction mixture (5 ml) consisted of 0.2 ml of egg albumin (from fresh hen's egg), 2.8 ml of phosphate buffered saline (PBS, pH 6.4) and 2 ml of varying concentrations of ethanol, ethyl acetate and hexane extract so that final concentrations become 50,100,150,200 µg/ml.

Similar volume of distilled water served as control. Then the mixtures were incubated at 37 ± 2ºC in a BOD incubator for 15 mins and then heated at 70ºC for 5 mins After cooling, their absorbance was measured at 660 nm by using vehicle as blank. Diclofenac sodium at the final concentration of (50,100µg/ml) was used as reference drug.

The percentage inhibition of protein denaturation was calculated by using the following formula:

$$\% \text{ inhibition} = 100 \times [V_t / V_c - 1]$$

Where,

V_t = absorbance of test sample.

V_c = absorbance of control.

RESULTS AND DISCUSSION

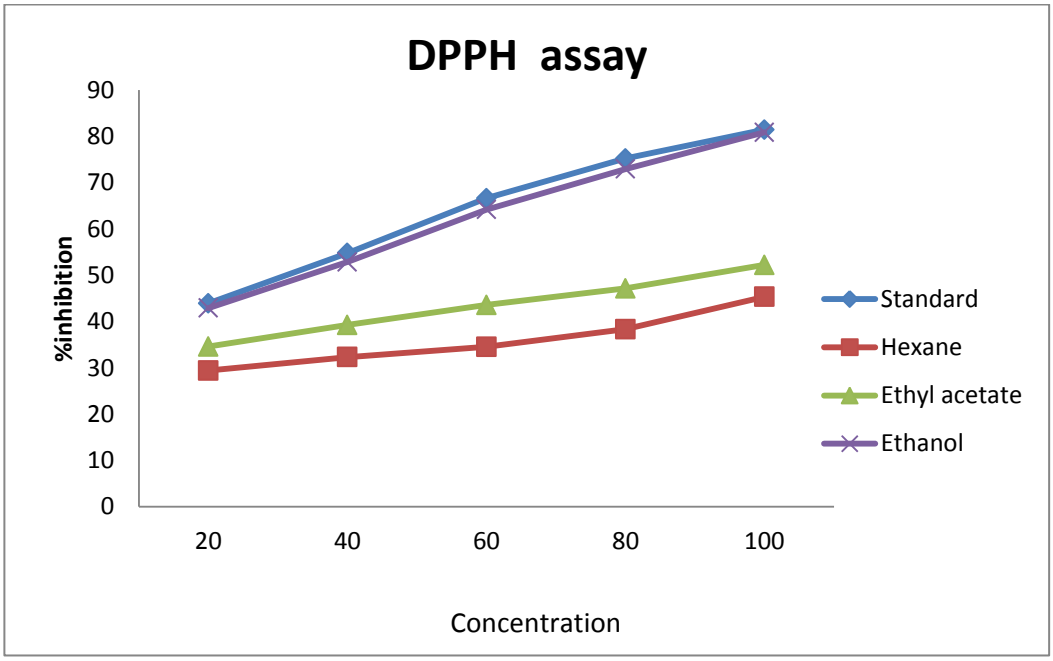
DPPH radical scavenging activity :

Various extracts were studied for its anti oxidant activity using 1, 2-diphenyl-2-picryl hydrazyl radical (DPPH).

Table 17: DPPH Radical Scavenging activity of various extract

S. No	Concentration (µg/ml)	% Inhibition			
		Standard (Butylated hydroxy Toulene)	Hexane	Ethyl acetate	Ethanol
1.	20	53.95	19.43	23.61	45.94
2.	40	64.81	29.32	31.28	59.86
3.	60	76.62	30.54	33.59	68.18
4.	80	85.21	31.34	43.16	76.95
5	100	91.45	37.34	52.23	88.87
IC_{50} (µg/ml)		10	152	97	25

Fig 20: DPPH Radical Scavenging activity of various extract



The DPPH is a stable free radical which produced deep purple colour by accepting the proton from any proton donor substance and widely used to test free radical scavenging effect. The reduction of DPPH decreases its absorbance at 517 nm due to colour change. The ethanolic extract of *Juticia procumbens* showed IC_{50} value (µg/ml) of 25 where as Hexane, Ethyl acetate showed Ic_{50} (µg/ml) value 152, 97, respectively. The IC_{50} (µg/ml) value of standard BHT was 10. Ethanolic extracts of *Juticia procumbens* was found to produce good scavenging activity in comparison to standard.

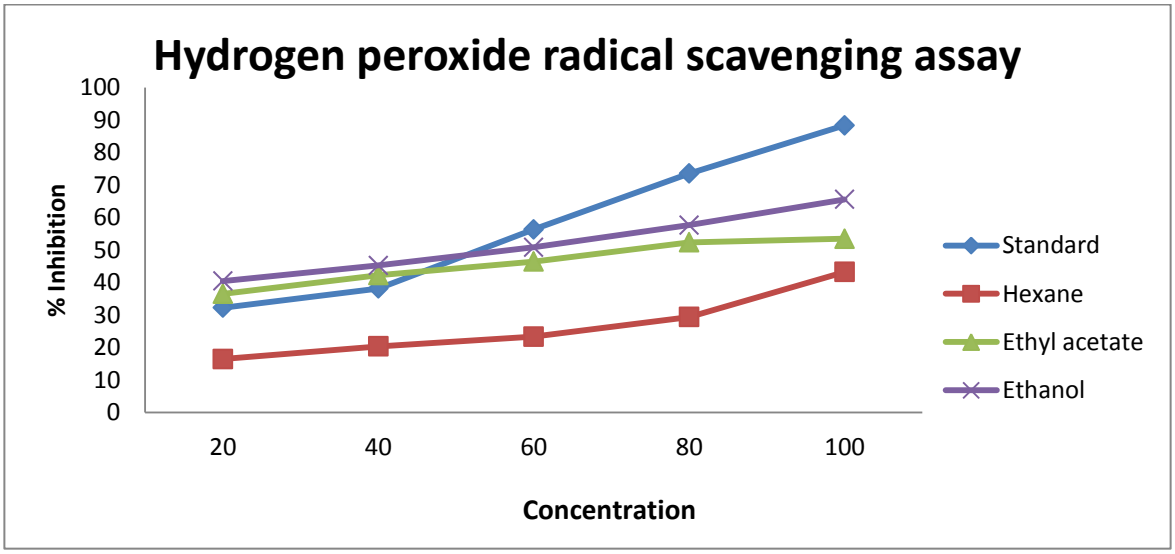
HYDROGEN PEROXIDE RADICAL SCAVENGING ACTIVITY:

The Hexane, ethyl acetate, ethanol and aqueous extracts were subjected to hydrogen peroxide radical scavenging assay and the results are tabulated below in Table 2 & Fig 2.

Table 18 : Hydrogen peroxide Radical Scavenging activity of various extract

S No	Concentration	% inhibition			
		Standard (Ascorbic acid)	Hexane	Ethyl acetate	Ethanol
1	20 µg/ml	32.23	16.43	36.51	40.43
2	40 µg/ml	38.13	20.32	42.23	45.65
3	60 µg/ml	56.34	23.34	46.43	50.81
4	80 µg/ml	73.32	29.34	52.34	57.65
5	100 µg/ml	88.34	43.23	53.43	65.56
IC_{50} (µg/ml)		47	127	77	54

Fig 21: Hydrogen peroxide Radical Scavenging activity of various extract



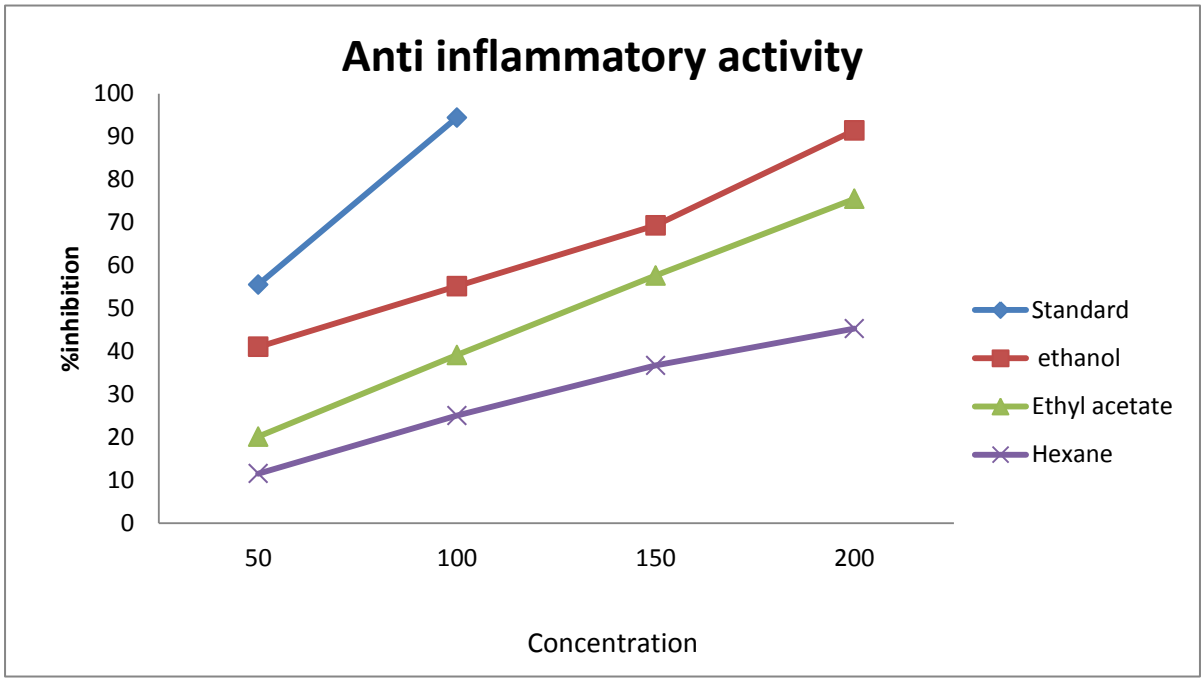
The ability of the extract to scavenge hydrogen peroxide was determined in this method. Absorbance is measured at 230 nm and IC_{50} (µg/ml) value has been calculated and was found to be 54 for Ethanol, Were as Hexane, Ethyl acetate showed the IC_{50} –value (µg/ml) of 127,77 respectively. IC_{50} value of standard Ascorbic acid was 47.

Table.19: *In vitro* anti-inflammatory activity of various extracts of *Justicia procumbens* Linn.,

S.No	Sample	Concentration(µg)	Absorbance	%inhibition
1.	Control	-	0.054	-
2.	Diclofenac (standard)	50 100	0.024 0.003	55.56 ±2.82 94.44 ±1.48
3.	Ethyl acetate	50 100 150 200	0.043 0.033 0.023 0.013	20.17 ±3.49 39.16 ±3.09 57.67 ±2.03 75.53 ±2.27
4.	Ethanol	50 100 150 200	0.032 0.024 0.017 0.004	41.05 ±1.81 55.17 ±2.06 69.34 ±1.46 91.43 ±1.58

Values are expressed by Mean ± SEM

Fig 22 Anti-inflammatory activity of various extracts of *Justicia procumbens* Linn.,



Ethanolic extracts produced higher inhibition of protein denaturation than the other extracts. At 100µg standard showed higher percentage inhibition of 94.44 ±1.48.The ethanolic extract showed higher percentage inhibition of 91.43 ±1.58 at 200 µg which was comparable with that of the standard.

10. FORMULATION OF HERBAL EYE OINTMENT[54-60]

10.1 MATERIALS AND METHODS

Extract used – Ethanolic extract

Dosage form – Ointment

Ointment base- Petrolatum base

Preservative – Benzalkonium chloride (0.01%w/v)

Eye ointment is advantageous than eye drops, since it was highly stable, improves drug contact time, increases drug release through diffusion on the membrane layer of eye.

Most preferred method for ointment preparation is by levitation method. The powder is first rubbed with a small quantity of the base to form a concentrated ointment base containing a finely divided powder uniformly distributed in it.

The concentrated ointment is then gradually diluted with remaining quantity of the base by rubbing with a spatula. The spatula should be of stainless steel with a long, broad flexible blade. When steel spatula cannot be used for reasons of reacting with certain drugs like iodine, salicylic acid, and mercury salts etc., a hard rubber spatula or a wooden tongue depressor may be used.

The preparation of the ointment was carried out in aseptic conditions.

The specified concentration of petrolatum base (5gm) with the ethanol extract were mixed in a porcelain slab over a period of time.

Two strength were considered for formulation – 0.2%w/w and 0.4% w/w which have 10mg and 20mg respectively.

EVALUATION OF OINTMENT

Physical Examination:

The Prepared ointment formulations were inspected visually for their colour, homogeneity, consistency.

Determination of pH:

2.5gm Ointment sample was taken in 100 ml dry beaker, 50 ml water was added to it. Beaker was heated on water bath maintained at about 60ºC to 70ºC for 10 minutes, cooled to room temperature, and then centrifuged at 3000 rpm for 10 minutes. The pH of water extract was measured by using pH meter. The pH measurements were done by using a digital type pH meter by dipping the glass electrode into the ointment formulation.

Extrudability:

Extrudability test is the measure of the force required to extrude the material from a collapsible tube when certain amount of force has been applied on it in the form of weight. In the present study the quantity in percentage of ointment extruded from the tube on application of certain load was determined. The extruadibility of prepared ointment formulation was calculated by using following formula.

$$\text{Extrudability} = \frac{\text{Amount of ointment extruaded from the tube} \times 100}{\text{Total amount of ointment filled in the tube}}$$

Physical Appearance:

Ointment formulations were white viscous preparation with a smooth homogeneous texture. Show Physical Appearance discussed in Table

Table 20 : Physical Appearance

S. No.	Formulation Code	Colour	Homogeneity	Consistency
1	F1	White	Good	++
2	F2	White	Excellent	+++

Determination of pH:

The pH of the ointment solution was measured with the help of pH meter. 0.5g of ointment was dissolved in 50ml of distilled water and stored for two hours. The measurement of pH each formulation was done in triplicate.

Table 21: pH of ointment

S. No.	Formulation Code	pH
1	F1	6.36±0.3
2	F2	6.27±0.1

Extrudability:

Ointments were filled into collapsible tubes after formulating them. The extrudability of the formulation has been checked.

Table 22 : Extrudability of ointment

S. No.	Formulation Code	Extrudability
1	F1	Easily Extrudable
2	F2	Easily Extrudable

Where F1- 0.2%w/w , F2- 0.4%w/w strengths of the oinments.

10.2 STABILITY TESTING OF OINTMENT

PROCEDURE

1. Keep several samples of the drug product at least three temperatures, such as 40 ºC, 50 ºC and 60ºC, 60 % RH,65 % RH, 75 % RH respectively.

2. Determine the drug content at all three storage points by taking a number of samples and take the mean drug content. We do this for a few weeks.

3. At each temperature we plot a graph between time and log percent drug remaining. If the decomposition is first order this gives a straight line. If it is zero order, percent drug remaining versus time will give a straight line.

4. Next we take the log K or log of reaction constant on axis and T x 0-3 on X axis and draw a best fit line. This line is the Arrhenius Plot, extrapolate this line to get k at 5 C and from this we calculate the shelf-life.

Topical preparations should be evaluated for appearance, clarity, color, homogeneity, pH, consistency, viscosity, particle size distribution , assay, degradation products, preservative and antioxidant content (if present), microbial limits/sterility, and weight loss (when appropriate).

11. PHARMACOLOGICAL EVALUATION OF HERBAL EYE OINTMENT

11. 1 MATERIALS AND METHODS

ACUTE EYE IRRITATION TEST IN RABBIT

Albino rabbits of either sex weighing between 2-3kg of 3 animals, twelve to twenty weeks old were selected for study.

Animals were individually housed in suspended cages, free access to diet and drinking water was allowed throughout the study. Provided with 17-23^{0}C temperature and 30- 70% relative humidity, 12 hours of continuous light and 12 hours darkness.

PROCEDURE: (Acute eye irritation test is designed as per the OECD guidelines 405).

Both eyes of selected test rabbits were examined with the aid of light source only animals free of ocular damage were used. A volume of 0.1ml or 100mg of the test material was placed into the conjunctiva sac of the right eye, formed by gently pulling the lower lid away from the eyeball. The upper and lower eyelids were held together for about one second immediately after treatment to prevent loss of the test material, and then released. The left eye remained untreated and was used for control purpose. Immediately after administration of the test material, an assessment of the initial pain reaction was made according to the six point scale.

After consideration of the ocular responses produced in the first treated animal, two addition animals were treated.

Assessment of ocular damage/ irritation was made approximately 1 hour, 24, 48 and 72 hours. If evidence of irreversible ocular damage is noted, the test material will be classified as corrosive to the eye.

Initial Pain Reaction

When the material (0.1ml or 100mg) is instilled in the eye there may be an initial local pain reaction. The reaction will be graded as follows:

TABLE 23. **Descriptive rating of initial pain reaction**

Class	**Reaction by animal**	**Descriptive Rating**
0	No response	No initial pain
1	A few blinks only, normal within one or two minutes	Practically no initial pain
2	Rabbit blinks and tries to open eye, but closes it	Slight initial pain
3	Rabbit holds eye shut and puts pressure on lids, may rub eye with paw	Moderate initial pain
4	Rabbit holds eye shut vigorously, may squeal	Severe initial pain
5	Rabbit holds eye shut vigorously, jump and try to escape	Very severe initial pain

*Upto class 2 its acceptable as non irritant as per OECD guidelines 405.

RESULTS AND DISCUSSION

TABLE 24: ACUTE IRRITANCY-PARAMETERS STUDIED

OBSERVATION	1 hr	24 hrs	48hrs	72hrs
Motor activity	+	+	+	+
Lacrimation	+	+	+	+
Loss of corneal reflex	-	-	-	-
Grooming	-	-	-	-
Excitation	+	+	+	+
Aggression	+	+	+	+

NOTE: +, - Indicates presence or absence

TABLE 25: INTIAL PAIN REACTION OBSERVED

Treatment	1hr	24hrs	48hrs
Animal 1	2	0	**0**
Animal 2	2	0	0
Animal 3	2	0	**0**

Since there were no irritation and behavioral changes observed. The eye ointment was considered as safe to administer

11.2. PHARMACOLOGICAL EVALUATION OF HERBAL OINMENT FOR INFLAMED EYE

MATERIALS AND METHOD

TURPENTINE INDUCED CONJUNCTIVITIS MODEL[61,62,64]

This is one of the most commonly used animal models for evaluating anti-inflammatory activity. Conjunctivitis is induced in rabbit's eye by instilling of 0.1microlitre of Turpentine liniment on the eye except for the positive control. Turpentine sudden irritation and inflamed condition of the eye leads to allergic conjunctivitis .The test drug and the standard drug will be administered for a period of 3 days (twice daily) and the desired parameters are observed.

Test drug – herbal ointment were given at the dose of $1/10^{th}$ (0.2%w/w) and $1/5^{th}$ (0.4%w/w) for 3 days.

EXPERIMENTAL ANALYSIS

A total of 30 adult Albino rabbits weighing (2-2.5 kg) were divided into 5 groups of 6 animals in each group.

TABLE 26: GROUPING OF ANIMALS

S.NO	GROUP	NAME OF THE GROUP	TREATMENT
1	I	Positive control	Treated with vehicle for 3 days
2	II	Negative control	Treated with turpentine liniment 0.1 microlitre
3	III	Standard	Treatment with betamethasone 0.05%w/w for 3 days
4	IV	Test drug1	Treatment with herbal eye ointment 0.2%w/w for 3days
5	V	Test drug2	Treatment with herbal eye ointment 0.4% w/w for 3days

Draize scale for Scoring Conjunctivitis

CONJUNCTIVAE

(A) Redness (refers to pelbebral and bulbar conjunctivae excluding cornea and iris)

Vessels normal	0
Vessels definitely injected above normal	1
More diffuse, deeper crimson red	2
Diffuse beefy red	3

(B) Chemosis

No swelling	0
Any swelling above normal	1
Obvious swelling with partial eversion of lids	2
Swelling with lids above half closed	3
Swelling with lids half closed to completely closed	4

(C) Discharge

No discharge	0
Any amount from normal	1
Discharge with moistening of the lids and hairs just adjacent to lids	2
Discharge with moistening of the lids and hairs just adjacent to lids a considerable area sround the eye	3

RESULT AND DISCUSSION

TABLE 27: CHANGES IN THE CHEMOSIS

Treatment	1st day	2nd day	3rd day	4thday
Group I (control)	0	0	0	0
GroupII (negative control)	3±0.043	3±0.017	2±0.032	2±0.017
Group III betamethasone 0.05%w/w	3±0.023	1±2.06**	1±1.58**	0±0.023***
Group IV Test ointment I 0.2%w/w	3±0.048	1±1.58	1±2.06*	0± 0.017***
Group V Test ointment II 0.4%w/w	3±0.013	1±2.27*	1±2.03**	0±0.032***

Values are expressed by Mean ± SEM(n=6). One way ANOVA followed by Dunnet's test *p<0.05, **p<0.01, ***p<0.001 were found to be significant when compared to negative control.

TABLE 28: CHANGES IN THE EYE DISCHARGE

Treatment	1st day	2nd day	3rd day	4thday
Group I (control)	0	0	0	0
Group II (negative control)	3±2.3	2±1.10	1±2.1	1±0.032***
Group III betamethasone 0.05%w/w	3±2.06	1±±3.09**	0±2.60***	0±0.90***
Group IV Test I ointment 0.2%w/w	3±2.6	1±2.06	0±0.032	0±0.50***
Group V Test II ointment 0.4%w/w	3±2.98±±	1±1.09**	0±2.1***	0±0.08***

Values are expressed by Mean ± SEM(n=6). One way ANOVA followed by Dunnet's test *p<0.05, **p<0.01, ***p<0.001 when compared to negative control.

TABLE 29 : CHANGES IN THE REDNESS

Treatment	1st day	2nd day	3rd day	4thday
Group I	0	0	**0**	0
Group II (negative control)	3±2.2	2±3.09	2±1.1	2±1.9
Group III betamethasone 0.5%w/w	3±2.4	1±1.5**	**1**±2.2***	0±1.90***
Group IV Test I ointment 0.2%w/w	3±2.0	1±1.9	**1**±0.04**	0±2.8***
Group V Test II ointment 0.4%w/w	3±2.3	1±3.09**	**1**±1.7***	0±0.50***

Values are expressed by Mean ± SEM(n=6). One way ANOVA followed by Dunnet's test *p<0.05, **p<0.01, ***p<0.001 when compared to negative control.

The results indicates that animals treated with test ointments and standard drug showed significant activity when compared to negative control. The conjunctivitis group of animals showed chemosis, eye discharge, redness in the eye. In the standard group and the group treated with ointment of 0.4%w/w all the parameters are brought back to the normal levels.

12. SUMMARY AND CONCLUSION

Allergic conjunctivitis is encountered in 15-40% of the population and is observed more frequently in spring and summer. Though there are several allopathic medicines available, people still rely on nature which has blessed human kind with abundant medicinal plants for prevention and cure of disease with less side effect and safety. Hence the present study is an attempt to formulate and evaluate herbal eye ointment on the leaves of *Justicia procumbens* Linn,.

PHARMACOGNOSTICAL STUDIES

The pharmacognostical studies on the leaf of *Justicia procumbens* Linn., was carried out, which showed the unique features of the leaf which is used to differentiate it from other species.

Macroscopical studies states the characteristic features of leaf like elliptic to oblong-ovate, 7-20mm long, 5-20mm wide, entire margin.

The microcopical studies of the leaf showed the presence of covering trichomes, epidermal glandular trichomes- sessile, capitate gland and diacytic stomata

Powder analysis of the leaf showed the presence of spongy parenchyma, lignified fibres, calcium carbonate crystals, covering and glandular trichomes.

Physiochemical studies various physiochemical constants were evaluated such as ash values, extractive values, foreign organic matter, foaming index, swelling index and loss on drying.

These pharmacognostical parameters evaluated are useful for the establishment of standards of leaf which is essential for its identity and purity.

Phytochemical studies

In phytochemical study, the powdered leaf was successively extracted with n-hexane, ethyl acetate and ethanol by using soxhlet apparatus.

Preliminary phytochemical investigation was done for the powdered leaf and all the extracts. It was found to contains flavonoids, alkaloids, phenolic compounds, steroids, etc,.

Quantitative estimation of the phytoconstituents is carried out for phenolic compounds and flavonoids.

Fluorescence analysis was done to find out characteristic fluorescent substance present in the powdered leaf and all the extracts, and no fluorescent substance was found.

High performance thin layer chromatography (HPTLC) was performed with ethyl acetate and ethanolic extract of leaf of *Justicia procumbens* Linn., and the finger print showed the presence of 12 peaks in the ethanolic extract and 7 peaks in ethyl acetate extract.

***In vitro* studies**

All the extracts were subjected to *in-vitro* antioxidant activity and *in-vitro* anti-inflammatory activity. The ethanolic extract showed the maximum antioxidant potential and maximum inhibition in the protein denaturation method. Hence the ethanolic extract was chosen for the *in-vivo* studies.

Pharmacological activity

Acute irritancy studies were carried out in the Albino rabbits as per the OECD guidelines 405. The Ethanol extract was found to be safe up to the dose of 100mg. Therefore $1/5^{th}$ of the dose (20mg) and $1/10^{th}$ of the dose (10mg) were selected.

Formulated herbal eye ointment were evaluated by *in vivo* method in Albino rabbits by using turpentine induced allergic conjunctivitis.

Dose 20mg (0.4%w/w) and 10mg(0.2%w/w). were instilled on eye of rabbits. It is compared with standard Bethamethasone (0.05%w/w) ointment. Various parameters were studied for the evaluation of anti-inflammatory activity. Eye ointment at the dose 20mg(0.4%w/w) showed significant anti-inflammatory effect on the conjunctivitis

induced rabbits which was comparable with that of the standard.

CONCLUSION

From the above studies it can be concluded that the Pharmacognostical standards generated will be useful for the proper identification of the plant that could be made use of, those who deal with the species and also in the quality assurance of the plant species. With the support of Phytochemical and *in-vitro* antioxidant and *in-vitro* Pharmacological studies, ethanolic extract was selected and subjected to *in-vivo* anti-inflammation on conjunctiva of the rabbit eye. Formulated herbal eye ointment with ethanolic extract at the dose of 20mg (0.4%w/w) showed significant activity which was comparable with that of the standard.

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DEAN OF FACULTIES

CERTIFICATE

This is to certify that Mr. A.BABU M. Pharm., II Year, Department of Pharmacognosy, College of Pharmacy, Madras Medical College, Chennai-03,had Submitted his Protocol (Part B Application) IAEC/MMC/12/2016 for the dissertation programme to the Animal Ethical Committee, Madras Medical College, Chennai-03.

TITLE: DEVELOPMENT AND EVALUATION OF HERBAL OINTMENT FROM *Justiciaprocumbens*Linn.,FOR INFLAMMATION OF EYE

The Animal Ethical Clearance Committee experts screened his proposal Number: IAEC/MMC/12/2016 and have given clearance in the meeting held on 21/11/2016 at Anatomydemo hall- III in Madras Medical College, Chennai-03. Hisstudy involves only Albino Rabbits

ANIMAL EXPERIMENTAL LABORATORY GOVERNMENT MADRAS MEDICAL COLLEGE
Dt. 21.11.2016
CHENNAI-3.

SIGNATURE

Dr. S.K. SEENIVELAN, B.V.Sc.,
Reg. No: 2175
SPECIAL VETERINARY OFFICER
ANIMAL EXPERIMENTAL LABORATORY
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INSTITUTE OF HERBAL SCIENCE
PLANT ANATOMY RESEARCH CENTRE

Prof.**P Jayaraman, Ph.D**
Director
Retd, Professor, Presidency College Chennai-5

AUTHENTICATION CERTIFICATE

Based upon the Organoleptic /macroscopic /~~microscopic~~ examination of fresh /~~market~~ sample, it is certified that the specimen given by A. Babu, Dept of Pharmacogn College of Pharmacy, MMC Chennai-3. is identified as below:

Binomial: Justicia Procumbens L.

Family: Acanthaceae

Synonym(s): Justicia Japanica Thunb.

Regional names: —

Reg.No of the certificate: PARC/2016/3274

References: Nair, N.C & Henry, A.N. Flora of TamilNadu, India I: ——— .1983.

Henry, A.N. *et al.* Ibid. ——— II: ——— .1987.

Ibid. ——— III: ——— .1989.

Ed:S.P.Ambasta,
The Useful Plants of India,
CSIR- Publication, 1986. J.S. GAMBLE 1921 Vol: II Pg: 1080

Date: 10.08.2016

(Prof.P.JAYARAMAN)
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Pharmacognosy
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(A Goverment of Puducherry Institution)

Department of Pharmacognosy,College of Pharmacy,Puducherry - 605 006

Approved by UGC Under Section 2(f) & 12(B),PCI,AICTE & BOME

Permanently affliated to Pondicherry University (A Central University)

RECENT TRENDS IN INDUSTRIAL PHARMACOGNOSY - 2016

RxTIP '16

Certificate

This is to certify that ~~Dr~~/Mr/~~Ms~~ BABU. A

of M. Pharm, COP- MMC

participated in the 5th National seminar on "Recent Trends in Industrial Pharmacognosy - 2016" Organised by Department of Pharmacognosy, College of Pharmacy, MTPG & RIHS, on 19th March 2016, at Puducherry and presented a paper in the Oral/Poster/e-Poster session titled

.....

with co-authors.....

V. Gopal

PROF. DR. V. GOPAL
REGISTRAR ACADEMIC
CONVENOR - RTIP'16

R

Dr. R. MURALI
DEAN - MTPG & RIHS
CHIEF PATRON - RTIP'16

ACCREDITED BY THE TAMILNADU Dr.M.G.R MEDICAL UNIVERSITY, CHENNAI WITH 05 CREDIT POINTS.

Introduction

Review of literature

Rationale for selection

Aim and Objective

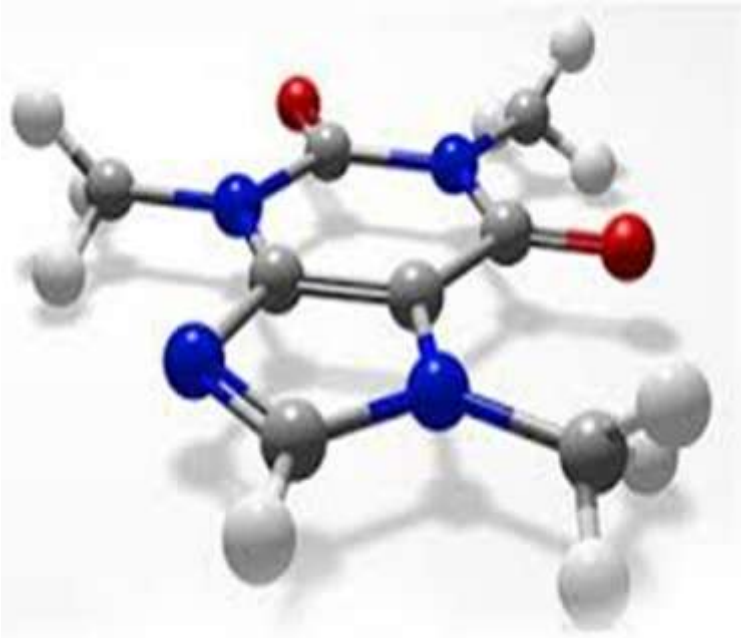
Plan of work

Summary and conclusion

References

Plant Profile

Pharmacognostical studies



Phytochemical studies

Selection of Active Extract

Pharmacological studies

Formulation